ANALYSIS, AND NUTRITIONAL EVALUATION FOR YOUNG CHICKS, OF SOME TOXIC FACTORS IN THREE NOVEL LEGUMES

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1987

CONTENTS PAGE

ABBREVIATIONS USED IN THIS THESIS

ACN:	acetonitrile
AME(c):	conventional apparent metabolisable energy
AME(n):	nitrogen corrected apparent metabolisable energy
CP:	crude protein
cv.:	cultivar
DHPOG:	3-o-glucuronide of 3,4-DHP
DM:	dry matter
dp:	particle diameter
ECD:	electrochemical detector; detection
EDE:	efficiency of deposition of energy
EE:	ether extract
EEC:	European Economic Community
EFC:	efficiency of food conversion
END:	efficiency of nitrogen deposition
ENR:	efficiency of nitrogen retention
FI:	food intake
GE:	gross energy
GC:	gas chromatography
GLC:	gas liquid chromatography
GR:	growth rate
HA:	haemagglutinating activity
HPLC:	high performance liquid chromatography
IEC:	ion exchange chromatography
IE HPLC:	ion exchange HPLC
JB:	jack beans
k':	chromatographic capacity factor
LLM:	*Leucaena* leaf meal
LS:	*Leucaena* seed
MeOH:	methanol
N:	column efficiency; number of theoretical plates
NAS:	National Academy of Sciences
NFE:	nitrogen free extractives
ODS:	octadecyl silane
OPA:	orthophthaldialdehyde
P:	peak separation parameter
PCAF:	pentacyanoammine-ferrate(III)
PC:	paper chromatography
PEC:	paper electrophoresis
PEG4000:	polyethyleneglycol (RAM = 4000)
PER:	protein efficiency ratio
PQ:	phenanthrene quinone
PTA:	phosphotungstic acid
PVP40:	polyvinylpyrrolidone (RAM = 40000)
RAM:	relative atomic mass
RGR:	relative growth rate
RPLC:	reversed phase liquid chromatography
Rs:	chromatographic resolution
RSD:	relative standard deviation
S:	solvent strength in RPLC
sem:	standard error of the mean
SSA:	sulphosalicylic acid
TCA:	trichloroacetic acid
THF:	tetrahydrofuran
TI:	trypsin inhibitor
TIU:	trypsin inhibitor units

TLC:	thin layer chromatography
T3:	triiodthyronine
T4:	tetraiodothyronine
2,3-DHP:	2,3-dihydroxypyridine
3,4-DHP:	3-hydroxy-4(1H)-pyridone

Other abbreviations assume the normal chemical and I.U.P.A.C conventions.

ABSTRACT

Two tropical legumes and a temperate legume were studied during the course of this work. These were *Leucaena leucocephala* (cv. Peru), *Canavalia ensiformis* (cv. unknown) and *Lupinus albus* (cv. Vladimir [Kievskji mutant]) respectively. The dried ground leaf obtained from *Leucaena leucocephala* (LLM), the seeds of *Canavalia ensiformis* (jack bean; JB) and *Lupinus albus* (lupin) were the materials used in the study.

Liquid chromatographic methods (HPLC) were developed for the analysis of mimosine and 3-hydroxy-4(1H)-pyridone (3,4-DHP) in LLM, *Leucaena* seed (LS) and chick excreta. Neither mimosine nor 3,4-DHP was detected in the serum of chicks fed LLM. Poor and variable recoveries of mimosine and 3,4-DHP were obtained when these were added to serum.

The analysis of canavanine in JB and the serum of chicks fed JB and canavanine, was also accomplished using HPLC. Canaline was not detected in any of the samples analysed by HPLC although recovery of added canaline to serum, JB and excreta was high. A small amount of what appeared to be canavanine was detected in lupin.

Saponins and tannins were found in all the legumes under study. Trypsin inhibitors were detected in all but the lupins.

Inclusion of LLM in chick diets reduced their performance. Addition of Fe(III), polyethylene glycol (RAM = 4000) and cholesterol to LLM diets improved chick performance almost to that of chicks fed control diets. Cooking LLM also improved chick performance. Addition of enzymes to LLM did not improve chick performance and did not improve the apparent metabolisable energy (AME) of LLM.

Dietary inclusion of mimosine or LS, to supply the same amount of mimosine as that from LLM, did not restrict chick performance to the same extent as LLM.

Inclusion of autoclaved jack bean (JB) in chick diets caused a severe reduction in chick performance. The reduction in performance was not matched by inclusion of canavanine at the same level as that from JB. The inclusion of extracted JB also reduced chick performance. Germination of the JB, prior to autoclaving and dietary inclusion, did not reduce canavanine levels nor was chick performance improved.

Addition of arginine to JB diets improved performance of chicks but additional lysine had no beneficial effect.

Lupin diets permitted chicks to perform much better than LLM or JB diets. Autoclaving made little difference to the AME of lupins although the results were probably confounded by the presence of Maillard reaction products. Addition of enzymes to lupins increased the concentration of lower molecular weight carbohydrates but only had a small beneficial effect on AME of lupins for chicks.

ACKNOWLEDGEMENTS

I wish to thank the numerous members of staff within the School of Agriculture who have shown interest in my work and who have encouraged and supported me during the course of this work. Special thanks are due to past and current members of the Agricultural Biochemistry Department for their support and encouragement throughout the period of the work presented here.

I gratefully acknowledge the assistance given by the staff of the Central Analytical and Trace Elements Department in obtaining the proximate analysis and mineral data.

Thanks are also due to the British Council for assistance in the provision of the Leucaena from Malawi and the jack beans from Mexico.

My sincere thanks are due to Dr. J.P.F. D'Mello for his advice and numerous hours of discussion regarding the conduct of the work presented here. I thank him for the hours he spent critically reviewing the first draft of this manuscript. In this context I apreciate the time my examiners will spend reading this thesis; I hope they find it interesting.

I wish to thank my parents and my brothers and sister for their interest and encouragement throughout the course of the work.

Many thanks are due to Mr. Scott for his surgical expertise which permitted me to continue this work unhindered.

My sincere thanks go to my patient and understanding wife, Fiona for her encouragement and for her assistance in reproducing the figures presented in this work.

The work contained in this thesis is my own original work and has not been submitted for a degree at any other University.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Legumes are the second most important group of plants utilised by humans. The major differences between legumes and the grasses, the most important and studied group of plants, is that legumes are nitrogen fixers, are dicotyledons and their seeds are produced in pods. The legume seeds, also known as pulses, are two to three times higher in protein content than grass seeds such as maize, wheat, rice, sorghum and rye thus making them an attractive source of protein for human and animal consumption.

Legumes exist symbiotically with bacteria of the *Rhizobium* spp. This symbiosis is essential for legumes to assimilate dinitrogen (N_2) directly from the atmosphere. The wide variety of *Rhizobium* spp. have a specificity for different legumes and the correct species must be present before invasion of the root system and subsequent nodulation, allowing nitrogen fixation and normal plant growth to occur. The fact that legumes fix nitrogen directly from the atmosphere means that nitrogenous fertilisers are unnecessary for normal plant growth; furthermore the soil is enriched in nitrogen.

There are about 18,000 species of Leguminosae spread throughout the world. However the two pulse legumes which have attracted most attention are soyabeans (*Glycine max*) and groundnuts (*Arachis hypogaea*) both for their oil and their food value. Of the remainder only about twenty are used in substantial quantities therefore there is ample scope for the study of unconventional or new legume crops and their potential uses.

Leucaena leucocephala, *Canavalia ensiformis* and *Lupinus albus* are examples of tropical forage, tropical pulse and temperate pulse legumes, respectively that have potential as high protein animal feeds.

The versatility of legumes is demonstrated by their ability to grow in a wide variety of conditions; from temperate to tropical high altitude to low altitude and from aquatic to semi arid conditions. The nutrient content of the soils has a considerable effect on the growth of legumes not solely because of the direct effects on the plants but because *Rhizobia* spp. have been shown to be susceptible to pH and mineral levels.

A major problem in the use of legumes as foods is that they contain a larger variety of toxic/anti-nutritional factors than any of the other plant families.

These factors vary widely in composition and include unusual toxic amino acids, proteins, glycoproteins, carbohydrates, alkaloids, polyphenolics and terpenoids. Their effects vary from inhibition of digestion to death. Fortunately some of the adverse effects can be reduced or removed by relatively simple methods such as germination, soaking in water and/or cooking. However these treatments are not always or completely successful. There is considerable scope for identifying what the toxic and/or antinutritional factors are in specific legumes. Furthermore considerable scope exists for identifying and elucidating the mechanism(s) associated with these compounds as well as in attempting to alleviate the problems due to the consumption of legumes by animals and man.

The presence, significance, effects, and methods of alleviating the effects on chickens, of some of the antinutritional factors mentioned above, will be discussed and data presented in this work. Details of methods of detection and quantification of some of these compounds will be presented and discussed throughout this work. The species of plant materials used, include *Leucaena leucocephala* leaf and seeds, *Canavalia ensiformis* and *Lupinus albus* seeds.

1.2 THE LEGUME FAMILY

The family Leguminosae is extremely large and varied containing between 17,000 and 18,000 species and 650 to 750 genera which are found in nearly every corner of the world (Purseglove, 1968; NAS, 1979; Polhill, Raven, Stirton, 1981; Allen and Allen, 1981). The various soils and climates in which legumes are found is also extremely varied (Adams and Pipolly, 1980).

The family Leguminosae consist of three sub-families; the Mimosoideae, the Caesalpinioideae and, by far the largest, the Papilionoideae. The mimosoideae are most commonly found as small trees or shrubs in semiarid tropical and sub-tropical areas of the world (Elias, 1981). The best known of the approximately 2800 species of this family are the acacias of which *Leucaena leucocephala* is a relative (NAS, 1979; Benge, 1981). The Caesalpinioideae are primarily trees found in tropical savannas and forests. This sub-family also contains about 2,800 members one of which is *Ceratonia siliqua* L. also known as the Carob bean (NAS, 1979; Duke, 1981).

The Papilionoideae, or Faboideae, consist of about 12,000 species most of which are herbs and which are distributed throughout the world. This group contain the *Lupinus* and *Canavalia* species as well as the more familiar *Arachis hypogaea* (L.) (groundnut) and *Glycine max* (L.) Merr. (soybean). Two of the more common forage legumes in this sub-family are *Medicago sativa* (L.) (lucerne) and *Trifolium repens* (L.) (white clover). The seeds of legumes date at least as early as 8500 BC. (Smartt, 1984).

1.2.1 SOME RELEVANT AND IMPORTANT PHYTOCHEMICAL PROPERTIES

There are a considerable number of phytochemical differences between the three sub-families but these are not always clear cut. Non protein amino acids frequently occur in high concentrations in the seeds of legumes (Harborne, 1984). Canavanine, a non protein amino acid, is found in 390 species of the Papilionoideae but is not reported to occur in any of the species of either of the other two sub-families (Birdsong, Alston and Turner, 1960; Bell, 1981; Fowden, 1981).

Although every species of legume which has been examined for flavonoids, has been shown to contain these molecules, the distribution of the various analogues is widespread. Papilionoideae infrequently contain any of the three most common flavonoids, myricetin, quercetin and kaempferol (Cowan, 1981) while they are readily found in arborescent Mimosideae, such as *Leucaena* (Lowry, Cook and Wilson 1984), and Caesalpinioideae. Mutagenic flavonoids such as quercetin and kaempferol (Friedman and Smith, 1984; MacGregor, 1984) are also found in various other plants including ferns and mosses (Swain, 1975; Seigler, 1981; Hahlbrock, 1981; Strack, Meurer, Wray, Grotjahn, Austenfield and Wiermann, 1984). Isoflavonoids, however, are primarily associated with the Leguminoseae (Hahlbrock, 1981; Harborne, 1984; Haslam, 1985) and these widely varying structural analogues (Haslam, 1985) are only found in the Papilionoideae (Cowan, 1981). The Papilionoideae group are the sole leguminous source of the two flavones luteolin and apigenin while the leucoanthocyanidin group of phenolics are rarely found in this family but are always found in the other two sub-families (Cowan, 1981).

Saponins have been reported to be present in all three sub-families (Seigler, 1981) as have the alkaloids. Vegetative parts as well as the seeds of some legumes are reported to contain saponins and have been found in Arachis, Phaseolus, Pisum, Vicia and *Lupinus* seeds (Nowacki, 1980). The quinolizidine alkaloids appear to be associated primarily with the Papilionoideae (Leonard, 1953; Walker and Dermer, 1981; Kinghorn and Smolenski, 1981; Grundon, 1984; Grundon, 1985) while pyrrolizidine alkaloids are characteristically found more widespread in the Leguminoseae (Haslam, 1985). The terpenoids, which include the saponins and gibberelins, are probably more abundant and varied in legumes than any of the other components mentioned above. Study of these has primarily been confined to commercially important plants. There is little information available on monoterpene distribution.

The limited studies on diterpenes in legumes indicate that there are few present in the Mimosoideae and Papilionoideae but those that are, are different from those detected in the Caesalpiniodeae (Langenheim, 1981). Triterpenes are more

prevalent in the Mimosoideae and the Papilionoideae than in the Caesalpiniodeae (Langenheim, 1981). The lack of information on this varied group of compounds may be due in part to the difficulty in their analysis. The increasing use of modern methods of analysis such as NMR, capillary gas chromatography, mass spectrometry and HPLC will increase the information available on the occurrence of these compounds, indeed examination of any recent issue of Phytochemistry confirms this premise.

1.3 NITROGEN FIXATION

Nitrogen fixing capacity of legumes and therefore establishment and growth is dependant on four important factors: 1, the inherited characteristics of the legume; 2, behaviour of *rhizobia* in the soil; 3, the variety of soil conditions and 4,the climate in which the plants are grown (Lowe, 1970; Bergersen, 1982; Ssali & Keya, 1986).

One of the major reasons why Legumes are important crop plants is because of their ability, in conjunction with *rhizobia*, to convert dinitrogen (N_2) directly from the atmosphere into a soluble utiliseable form by the plant. Legumes, however, are not unique in their ability to utilise atmospheric nitrogen in symbiosis with plants. Recently it has been demonstrated that millet (*Pennisetum americanum* (L.) Leeke), innoculated with nitrogen fixing bacteria, increased nitrogen uptake and the nitrogen content of the seed (Wani, Chandrapalaiah and Dart, 1985).

The specificity, soil conditions, type and function of *rhizobium* species is the subject of numerous reports (Skerman, 1977; Postgate, 1982; Bergersen, 1982). The intriguing mechanism by which nitrogen fixation in legumes occurs is under intense study and has been discussed elsewhere (Bergersen, 1982; Jolivet and Mosse, 1983; Sprent and Minchin, 1985).

1.4 GROWTH CONDITIONS FOR LEGUMES

Pasture and pulse legumes require soil which permits roots to penetrate relatively easily and deeply. The soil also requires to be free draining, able to sustain *rhizobium* spp. but need not be abundant in its content of nitrogen (Smartt, 1976). Good growth is obtained when the soil is fairly well aerated and the water content is between 75% to 85% of the water holding capacity (Skerman, 1977). Optimum pH conditions are between 6.5 and 7.5 in medium loam (Cooper, Wood and Holding, 1983). Temperate legumes are especially sensitive to low pH while many tropical legumes thrive in soils with a pH as low as 4 provided other nutritional requirements are met (Skerman, 1977; Lowe, 1970).

Temperature effects vary depending on the species of legume and its associated *rhizobia*. Movement of temperature from the optimum inhibits nitrogen fixation by *rhizobia* and therefore plant growth. Survival of plants is possible at temperatures as low as -15°C for temperate legumes while tropical legumes survive temperatures above 45°C (Smartt, 1976; Skerman, 1977; Hardwick, 1983; Ollerenshaw, 1983). As well as being responsive to temperature, legumes also respond variably to period of daylight where tropical legumes tend to develop more rapidly with a short photoperiod and vice-versa for temperate legumes (Hadley, Summerfield and Roberts, 1983).

The conditions under which legumes grow are likely to have a major effect on their nutritional or antinutritional properties either by reducing the quality of the nutritionally valuable components or by increasing the quantity of antinutritional components (Skerman, 1977; Oram, David, Green, Read, 1979; Bressani and Elias, 1980).

1.5 USES OF LEGUMES

The use of legumes are very varied. One of the major roles of legumes is as a food source for humans and animals. Most legumes used in this way belong to the Papilionodeae and are grain producing. Others are leafy temperate or tropical forage crops which can be grown by themselves or with grasses. In some cases the leaves, seeds and tubers can be used for food although a compromise has to be reached in terms of what should be used and at what time it should be harvested. This is necessary because if leaf is harvested seed production may drop and, obviously, if the tuber is harvested the crop is unable to grow. The winged bean (*Psophocarpus tetragonolobus* (L.) DC.) is a lesser known tropical legume, but of increasing importance, in which the whole plant can be used while the pea (*Pisum sativum* L.) is a well known temperate crop where leaf and seed is used (NAS, 1981; Smartt, 1985; Eagleton, Khan and Erskine, 1985; Davies, Berry, Heath and Dawkins, 1985). Forage crops can be browsed, dried or ensiled for feeding at a more suitable time of year (Skerman, 1977). The advantage of feeding forage legumes, either solely or as a mixture, is that the protein level of the diets is increased due to the higher protein content of legumes compared to grasses.

The use of legumes as rotation crops, anti erosion crops and for ornamental purposes has been successful but intercropping these with other crops has had only limited success (NAS, 1979; 1983; Putnam, Herbert and Vargas, 1985).

Detailed uses of legumes can be found in the literature (Purseglove, 1968; Skerman, 1977; Duke, 1981).

1.6 PRODUCTION OF LEGUMES

Legumes provide a major source of animal and human food throughout the world and production rates are increasing faster than for cereals. The rate of increase in production of cereals and pulses from 1980 to 1984 was 14.9 and 18.3% respectively (FAO 1982; 1984).

The production of the different legumes occur to varying extents in different areas of the world. Tropical countries are the major suppliers of legume seed crops, however the rate of increase in production in Western Europe and Oceana from 1980 to 1984 is noteable (FAO, 1980; 1984). The increased interest in legume crops in Western Europe is, no doubt, partly due to the pressures on the E.E.C. cereal producers who are looking for alternatives to fit in with their cereal rotations. Less common crops such as lupins, cowpeas and guar have estimated productions of approximately 0.5, 2 and 1 M tonnes per annum (Verma, 1977; Pate, Williams and Farrington, 1985; Steele, Allen and Summerfield, 1985)

Data for the production of less conventional legumes such as the winged bean, jack bean and *leucaena* are not available. As the potential of these and other underexploited legumes is realised and with the development of better production systems accompanied by further research on the production of different cultivars with higher yields (Goldsworthy, 1982), they will begin to have an influence on world trade. The study and modification of present farming techniques is also likely to enhance legume production.

The production of better quality products and better marketing techniques is also likely to increase the production and use of legumes (Bressani, 1975; Abbot, 1982). If the developing countries can substitute locally grown crops such as jack beans and *leucaena* for crops like soyabean which they currently import, then considerable savings in foreign currencies may be made.

1.7 COMPOSITION AND NUTRITIONAL ATTRIBUTES

The supplementation of grass diets for ruminants, with forage foliar legumes such as Lucerne and clovers is well documented and practised. Inclusion of such legumes in ruminant diets is beneficial in two ways: 1. because of the higher protein content of legumes, protein intake is increased and; 2. the nutritive value of species such as clover and lucerne is superior to grasses ('t Mannetje, O'Connor, and Burt, 1980; Thomson, 1984; Horner, Bush, Adams and Taliaferro, 1985). Monogastric animals, on the other hand, respond adversely when fed untreated legumes such as lucerne and some of its fractions (Cheeke, 1976; Cheeke, Pedersen and England, 1978; Hegsted and Linkswiler, 1980; Ueda, Ohshima and Kamada, 1986). Lucerne and clover fractions have, however,

TABLE 1.7.1 PROXIMATE ANALYSIS OF SOME LEGUMES
(g kg^{-1} dry matter)

Plant	Ash	CP£	Lipid	Fibre*	NFE+	ref
(forage)						
Medicago sativa	97	200	38	201	464	1
Trifolium repens	127	202	24	229	418	1
Trifolium pratense	106	167	31	308	388	1
Centrosema pubescens	83	212	31	301	373	2
Glycine wightii	168	210	37	375	210	1
(seed)						
Arachis hypogaea	23	348	491	31	107	1
Pisum sativum	33	244	20	48	655	1
Vicia faba	39	283	24	88	566	1
Lens culinaris	24	296	31	32	617	5
Cicer arietinum	34	206	16	67	600	5
Vigna unguiculata	44	265	18	70	603	10
Glycine max	47	394	184	62	313	6
Phaseolus vulgaris	41	261	18	66	614	5
Phaseolus lunatus	34	214	14	43	611	7
Vigna radiata	30	256	30	35	649	5
Cajanus cajan	49	211	37	64	639	10
Psophocarpus tetragonolobus	33	380	150	123	308	1,9
Cyamopsis tetragonolobus	60	450	57	101	332	4
Leucaena leucocephala	40	314	65	159	422	8
Ceratonia siliqua	30	88	5	89	788	3

£ :crude protein = N* 6.25 * : crude fibre + : nitrogen free extractives obtained by subtraction.

refs. 1 : McDowell, Conrad, Thomas, & Harris.(1974). 2 : Skerman (1977). 3 : Fredella, Mallosini & Martillotti. (1983). 4 : Verma,(1977). 5 : Bressani & Elias. (1980). 6 : D'Mello, Acamovic & Walker. (1983). 7 : Lyman, Baudoin & Hidalgo. (1985). 8 : Lee. (1981). 9 : Khor, Tan & Wong, (1982). 10 : Nwokolo and Oji, (1985).

produced enhanced growth rates when fed to broiler chickens (Korniewicz, Gwara, Mazanowska and Kaczmarek, 1981). The adverse effects of forage legumes, when fed to monogastrics, may be attributed to their higher fibre content and the presence of antinutritional factors, compared to grains such as maize. Ruminants are not completely free from adverse effects when fed forage legumes ('t Mannetje, O'Connor and Burt, 1980).

The obvious difference between forage and seeds from legumes is that the seeds have a much lower fibre content (Table 1.7.1) and are therefore generally more digestible. In general the legumes are characterised by their high protein content. The carbohydrate (soluble and obtained by difference in Table 1.7.1) content, in seeds especially, is also high. Lipid tends to be relatively low. There are three obvious exceptions in the above list, groundnut, soyabean and winged bean which have lipids close to those of the oilseeds (Table 1.7.2). The oilseeds have considerably lower carbohydrates but higher fibre than legume grains. The carbohydrate content of the three cereals (Table 1.7.2) is slightly higher than legume grains but protein is at least half. Legume seeds are reported to be good sources of phosphorous, iron and some vitamins and the lipid tends to be unsaturated (McDowell, Conrad, Thomas and Harris, 1974; Bressani and Elias, 1980; Norton, Bliss and Bressani, 1985). Variation in nutrient content, particularly protein, in legumes is influenced by the environment in which they grow (Bressani and Elias, 1980). A recent study of potentially useful legumes in Africa, however, has shown that seeds of Mimosoideae are higher in nitrogen and minerals than those of the Caesalpinoideae family (Balogun and Fetuga, 1986).

The major storage carbohydrate in legume seeds is starch but significant amounts of lower molecular weight carbohydrates are also found. These include glucose sucrose, raffinose, verbascose, stachyose as well as galactomannans (Lourdes, Bianchi, Silva and Braga, 1984; Reddy, Pierson, Sathe and Salunkhe, 1984; Carre, Brillouet and Thibault, 1985). Some of the carbohydrates in legumes are of no nutritional value and, in some cases are detrimental (Bressani and Elias, 1980; Arora, 1983; Norton, Bliss and Bressani, 1985; Struthers, 1986). Recent studies have shown that treatments such as germination, soaking in water or sodium bicarbonate solution reduced the levels of low molecular weight carbohydrates which are present in five legume seeds (Jood, Mehta, Singh and Bhat, 1985).

Legumes contain a vast variety of proteins some of which are anti-physiological in nature when consumed by animals; heat treatment of these legumes reduce these anti-physiological effects and thus allow the proteins to become utilisable by the animal which has consumed them. In some cases washing has improved the nutritional quality of legumes (Vohra, 1983). The majority of the proteins are

TABLE 1.7.2 PROXIMATE ANALYSIS OF THREE MAJOR CEREAL AND OILSEED GRAINS (g kg^{-1} DM)

Grain	Ash	CP	Lipid	Fibre	NFE	ref
Maize	14	101	45	20	820	1
Wheat	18	143	19	34	786	1
Sorghum	20	125	34	22	799	1
Rapeseed	43	201	422	76	257	2
Sunflower	33	181	364	258	164	2
Cottonseed	38	229	241	205	287	2

CP : crude protein = N * 6.25; NFE : nitrogen free extractives.

Ref. 1 : Crampton & Harris, (1969). 2 : McDowell, Conrad, Thomas and Harris, (1974).

TABLE 1.7.3 INDISPENSABLE AMINO ACID CONTENT OF SOME GRAINS (g kg^{-1} DM)

Seed (ref.)	Met	Cys	Lys	Arg	Trp	Thr	Phe	Val
Groundnut(1)	3.9	4.5	12.2	39.0	3.9	8.9	17.3	14.5
Faba bean(2)	2.4	4.2	17.3	28.1	2.6	10.3	12.4	13.3
Soya bean(3)	4.1	3.7	19.5	24.3	---	14.7	20.5	19.7
Winged bean(3)	3.2	5.0	28.4	26.9	3.2	14.0	16.5	17.6
Guar(4)	4.9	6.0	18.6	12.0	4.1	14.0	16.3	15.6
Maize(5)	2.7	2.2	3.0	5.0	0.5	3.7	5.2	5.0
Wheat(5)	2.4	2.6	3.6	6.0	1.4	3.6	5.6	5.3
Sorghum(6)	1.4	1.7	3.2	4.3	1.1	3.0	5.0	6.0
Rice(6)	1.2	0.7	3.2	9.3	1.8	2.6	3.4	4.7
ARC(7)*	4.0	3.5	9.0	8.5	1.7	6.0	7.0	8.0

Met : methionine; Cys : cystine; Lys : lysine; Arg : arginine; Trp : tryptophan; Thr : threonine; Phe; phenylalanine; Val : valine.

Refs. 1 : Paul & Southgate. (1978). 2 : Bond, Lawes, Hawtin, Saxena & Stephens.(1985). 3 : D'Mello, Acamovic & Walker. (1983). 4 : Verma (1977). 5 : Blair, Harber, McNab, Mitchell & Scougall. (1981). 6 : McDowell, Conrad, Thomas & Harris. (1974). 7 : ARC, (1975).

* : Amino acid requirements of the young chick as estimated by ARC (1975).

reported to be storage proteins consisting primarily of globulins and considerably smaller amounts of glutelins and albumins (Gupta, 1983; Norton, Bliss & Bressani, 1985).

The lower digestibility of legume protein compared with protein from animal sources is well known (Bressani, 1975; Eggum & Jacobsen, 1976; Tobin & Carpenter, 1978; Minson, 1982; Sandaradura & Bender, 1985; Norton, Bliss & Bressani, 1985) however cereal proteins have a similar digestibility to legumes. The lower digestibility of legume proteins has been attributed to a number of possible factors including the presence of thermally stable and residual proteinase inhibitors, the effect of the heat pretreatment, the tertiary protein structure and the presence of polyphenolic compounds (Hewitt & Ford, 1982; Norton, Bliss & Bressani, 1985). It has also been suggested that the lower protein digestibility may be due to an increased loss of endogenous nitrogen rather than to a reduction in absorption of dietary nitrogen (Bender & Mohammadiha, 1981). There is considerable variation in the digestibility of proteins from different legume sources which can be attributable to the type of protein and/or the pretreatment or lack of it (Verma, 1977; Tobin & Carpenter, 1978; D'Mello, Acamovic & Walker, 1983; Gupta, 1983; Sandaradura & Bender, 1985).

Although the amino acid content of legumes is considerably higher than that of cereals (Table 1.7.3) there is an imbalance of amino acids. This imbalance is particularly obvious if the sulphur amino acids, methionine and cystine are compared with the others and a comparison is made between the ratio of amino acids in the ARC estimates of requirements (Table 1.7.3). Recently some legume seeds have been shown to have considerably higher concentrations of sulphur amino acids than conventional seeds (de Lumen, Becker and Reyes, 1986). The low content of sulphur amino acids in the protein of legumes however, may be due to underestimates of these caused by difficulties of analysis (Newton & Hill, 1983; Williams, 1986). Therefore reports that legumes are deficient in sulphur amino acids are not necessarily correct (Wyckoff, Vohra and Kratzer, 1983).

Tryptophan is usually present in adequate amounts in legumes while lysine, which is obviously limiting in cereals, is abundant provided the heat pretreatment is not sufficiently harsh as to reduce the availibility. A combination of legume protein and cereal protein is likely to give diets adequate levels of available indispensable amino acids. In some cases supplementation with sulphur amino acids and/or animal proteins is desirable.

In general, legumes, excluding those with high oil content (Table 1.7.1), are considered as food protein sources rather than energy sources. However legumes, particularly grain legumes, have reasonably high apparent metabolisable energy values (AME) although there is some considerable variation (McDowell, Conrad,

Thomas and Harris, 1974). This variation in AME occurs between legume types as well as within species (Tobin and Carpenter, 1978; D'Mello and Acamovic, 1982; D'Mello, Acamovic and Walker, 1983; Newton and Hill, 1983; D'Mello, Acamovic and Walker, 1985). Metabolisable energy is dependant on pretreatment of the legume prior to consumption as well as area of growth, time of year of planting and stage of harvesting (Verma, 1977; Minson, 1982; Newton and Hill, 1983; Nwokolo and Oji, 1985). The variation of AME is most likely due to the type of carbohydrate and lipid present in the legume (Minson, 1982; Norton, Bliss and Bressani, 1985). In some cases the AME of legumes is about the same as some cereals (McDowell, Conrad, Thomas and Harris, 1974; D'Mello, Acamovic and Walker, 1983; 1985).

1.8 ANTINUTRITIONAL AND TOXIC COMPONENTS IN LEGUMES

The antinutritive, non nutritive and toxic compounds vary in nature from high molecular weight polymers such as proteins and polysaccharides to small monomers or oligomers such as amino acids and phenols. These compounds can be heat stable or heat labile (Tables 1.8.1 & 1.8.2). Some of these compounds tend to be present as storage components of nitrogen and energy. Because of the deleterious effects in animals when some of these compounds are ingested it has been postulated that they protect the plants from infection and predation and therefore genetic selection through time has produced a proliferation of plants containing these physiologically active and deleterious compounds (Bell, 1981a; Haslam, 1985; Norton, Bliss and Bressani, 1985; Harborne, 1986). The premise that these compounds acts as part of the plant defence mechanism is supported by the increased level of protease inhibitors found at wounds in plants (Liener and Kakade, 1980) while lectins have been associated with the protection of plants from phytopathogens (Lis and Sharon, 1980). Lectins have also been shown to be involved in nitrogen fixation which assists in accounting for the specificity associated with *rhizobia* and a particular plant (Lis and Sharon, 1980). The role of antiphysiological compounds in plants has been eloquently summarised in what is basically a modified form of the Le Chatelier principle as applied to a biological system (Bell, 1978a).

The physicochemical properties of some of these compounds may, however, be modified to varying extents, thermally. Table 1.8.1 summarises the susceptibility of some of these compounds to heat while compounds not susceptible to heat treatment are shown in Table 1.8.2.

The decomposition of thermolabile proteins in beans have been shown to follow first order kinetics thus indicating that initial treatment will have the greatest effect (Buera, Pilosof and Bartholomai, 1984). Destruction of the majority of the heat labile components occurs within the first 15 to 30 min of heating but is

TABLE 1.8.1 SOME ANTIPHYSIOLOGICAL COMPOUNDS FOUND IN PLANTS AND THEIR SUSCEPTIBILITY TO HEAT.(HEAT LABILE)

CLASS OF COMPOUND	Ref.	EXAMPLE	TYPE	PLANT SOURCE
haemagglutinin (lectin)	(1,2,3)	concanavalin-A	protein	*Canavalia ensiformis*
"		phaseolus lectin	glycoprotein	*Phaseolus vulgaris*
"		ricin	glycoprotein	*Ricinis communis*
protease inhibitors	(4,5)	soyabean	protein	*Glycine max*
"		favabean	protein	*Vicia faba*
goitrogens	(6,7)	soya-goitrogen	oligopeptide	*Glycine max*
cyanogens	(8,9)	phaseolunatin	cyanogenic glycoside	*Phaseolus lunatus*
anti-vitamins	(10,11)	anti-D	unknown	soyabean
"		anti-E	unknown	*Phaseolus vulgaris*
carbohydrase inhibitors	(12,16,17)	amylase inhibitors	glycoprotein	*Phaseolus vulgaris*
tannins	(12-15)	general enzyme	polyphenolic polymer	*Vicia faba*
"		inhibitor ?	"	*Sorghum vulgare*
"			"	*Psophocarpus tetragonolobus*

REFERENCES

1: Jaffe (1980); 2: Liener (1980); 3: Richer, Carriere, Blythman & Vidal (1982); 4: Liener & Kakade (1980); 5: Newton & Hill (1983); 6: Konijn, Edelstein & Guggenheim (1972); 7: Konijn, Gershon & Guggenheim (1973); 8: Montgomery (1980); 9: Conn (1981); 10: Liener (1980); 11: Liener (1983); 12: Liener(1980a); 13: McLeod (1974); 14: Buckley, Devlin & Marquardt (1983); 15: Tan, Wong & de Lumen,(1984); 16: Galagher & Schneeman, (1984); 17: Lajolo & Filho, 1985.

TABLE 1.8.2 SOME ANTIPHYSIOLOGICAL COMPONENTS IN PLANTS AND THEIR STABILITY ON HEATING (HEAT STABLE).

CLASS OF COMPOUND	Ref.	EXAMPLE	TYPE	PLANT SOURCE
alkaloids	(1-4)	sparteine	quinolizidine	*Lupinus spp.*
amino acids	(5,6)	canavanine	aliphatic guanidino	*Canavalia* & *Medicago ssp.*
"	(7,8)	indospicine	aliphatic imino	*Indigofera spicata*
"	(9,10)	mimosine	pyrrolidone	*Leucaena leucocephala*
cyanogens	(11,12)	linamarin	cyanogenic glycoside	*Phaseolus* & *Trifolium spp.*
saponins	(13,14)	medicagenic acid	glycosidic triterpenoid	*Medicago sativa*
"		?	?	*Lupinus Pisum* & *Vicia*
flavonoids	(15-17)	genistien	isoflavone	*Trifolium spp.*
"		kaempferol	flavonoid	*Leucaena spp.*
"		naringenin	flavanone	*Acacia spp.*
"		condensed tannins	polymeric flavonols	*Sorghum vulgare*
phenolics	(18-21)	hydrolysable tannins	polygalloyl esters	*Medicago sativa*
pyrimidine glycosides	(22,23)	vicine convicine	pyrimidine glycosides	*Vicia sativa*
carbohydrates	(24)	guar gum	galactomannan	*Cyamopsis tetragonoloba*
goitrogens	(25)	DHP	hydroxy-pyridone	*Leucaena spp.*
"	(26-28)	glucosinolates	progoitrin	*Brassica spp.*

REFERENCES.
1: Waller & Dermer (1981); 2: Kinghorn & Smolenski (1981); 3: Hatzold, Elmadfa, Gross, Wink, Hartmann & Witte (1983); 4: Priddis (1983); 5: Bell (1972); 6: Natelson (1985); 7: Hegarty & Pound (1969); 8: Hegarty & Pound (1970); 9: Roy (1981); 10: Acamovic, D'Mello & Fraser (1982); 11: Montgomery (1980); 12: Conn (1981); 13: Nowacki (1980); 14: Birk & Peri (1980); 15: Wong (1975); 16: Hahlbrock (1981); 17: Lowry, Cook & Wilson (1984); 18: Haslam (1979); 19: Haslam (1981); 20: Kumar & Singh (1984); 21: Sosulski & Dabrowski (1984); 22: JSFA 783 23: Arbid & Marquardt (1985); 24: Verma (1977); 25: Hegarty, Lee, Christie, Court & Haydock (1979); 26: Tookey, VanEtten & Daxenbichler (1980); 27: Larsen (1981); 28: Spinks, Sones & Fenwick (1984).

highly dependant on the moisture content and the temperature at which the sample is treated (Grant, More, McKenzie and Pusztai, 1982; Buera, Pilosof and Bartholomai, 1984). The use of microwave treatment has shown that heat treatment for less than 3 min destroys about 90% of the trypsin inhibitor activity in soya beans (Hafez, Singh, McLellan and Monroe-Lord, 1983).

It is imperative to note that heat pretreatment of foodstuffs containing antiphysiological compounds may reduce or eliminate the physiological problem associated with, say, a particular protein and it may even enhance the nutritional characteristics of the protein (Bressani and Elias, 1980; Sandaradura and Bender, 1985) but in doing so the treatment may destroy, partially or fully, other nutritionally desirable characteristics such as vitamins, pigments, amino acids and lipids. The availability of such compounds as amino acids and carbohydrates may also be reduced and toxic compounds such as lysinoalanine, may be produced (Woodard and Alvarez, 1967, Carpenter and Booth, 1973; Yannai, 1980; Friedman, Gumbmann and Masters, 1984; Tressl, Grunewald, Kersten and Rewicki, 1986).

The conjugation of carbohydrates and amino acids/proteins in foodstuffs to produce melanoidins, thus reducing the availability of the carbohydrates and amino acids, is dependent on moisture content, period of treatment and temperature (Benzing-Purdie, Ripmeester and Ratcliffe; 1985).

In some cases animals reduce the toxicity of some of the deleterious compounds, which are sometimes known as xenobiotics, present naturally or by sample treatment, either by degradative reaction or by conjugation (Hegarty, Schinckel and Court, 1964; Millburn, 1978; Tangendjaja and Wills, 1980; Reichert, 1981).

1.8.1 Protease Inhibitors

Protease (or proteinase) inhibitors are, primarily, a varied series of proteins (RAM 6000-25000; Laskowski and Kato, 1980; Hafez and Mohamed, 1983) which are found in a large variety of legumes, primarily in the seed cotyledon (Valdebouze, Bergeron, Gaborit and Delort-Laval, 1980; Gatehouse and Boulter, 1983). They are partially or completely destroyed by heat (Table 1.8.1; Liener, 1978); this destruction being enhanced by the presence of moisture and thiols (Liener, 1978; Friedman, Grosjean and Zanhley, 1982). Such treatment also enhances the nutritive value of the treated proteins.

It has been shown that there are as many as ten proteinase isoinhibitors of RAM of about 6000 (ie. Bowman-Birk) in eight different strains of soyabeans (Tan-Wilson, Cosgriff, Duggan, Obach and Wilson, 1985). There are also a number of protease inhibitors of higher molecular weight but with a lower proportion of disulphide bonds (ie. Kunitz inhibitors; Liener & Kakade, 1980). Protease

inhibitors of various activities have been isolated and characterised from different legumes. The active sites, that is the areas within the protease inhibitors which have a particularly high affinity for the proteases, have also been identified and characterised. The amino acid pattern around these sites have a close similarity (Liener and Kakade, 1980). Trypsin inhibitors, the most commonly studied protease inhibitors, have either lysine or arginine at their active sites while leucine or phenylalanine have been identified as the active sites of chymotrypsin inhibitors and alanine, in elastase inhibitors (Liener and Kakade, 1980).

Polyphenolics have also been implicated in protease inhibition (Griffiths and Moseley, 1980; Fernandez, Elias, Braham and Bressani, 1982; Tan, Wong and de Lumen, 1984), however the effects of these are discussed later in this chapter. Glycoproteins from lupins have also been shown to inhibit trypsin. The cause of the inhibition has been attributed to steric hindrance due to the presence of the carbohydrate moiety on the protein (Semino, Restani and Cerletti, 1985). This steric hindrance may also partially account for the inhibition caused by proteins from other plants. There are also some, as yet uncharacterised, non-protein protease inhibitors present in soya beans and winged beans (Hafez and Mohamed, 1983). Recent studies (Oste, Dalqvist, Sjostrom, Noren and Miller, 1986) have shown that Maillard reaction products have also inhibited trypsin and some other enzymes.

Despite considerable efforts the precise mechanism by which protease inhibitors exert their effects when ingested by animals has not been elucidated. Recently it was reported (Tan-Wilson, Cosgriff, Duggan, Obach and Wilson, 1985) that inhibition is caused by the inhibitory proteins forming complexes with mammalian pancreatic serine proteinases. The enzyme-inhibitor complex is reported to be (Ryan, 1981) similar in nature to the enzyme-substrate transition complex which is fixed in that state and has not progressed in the usual way to give hydrolysis.

One of the results of ingestion of proteinase inhibitors is that the pancreas of the animal increases in size, due primarily to hypertrophy and hyperplasia, and pancreatic secretions also increase (Liener, 1978; Gallaher and Schneeman, 1984). The increased secretion of trypsin, caused by ingestion of trypsin inhibitor, is reported to induce the increase in pancreas size. Pancreatic secretion of trypsin is controlled by the concentration of intraluminal trypsin which inhibits the release of the hormone cholecystokinin. Therefore if trypsin is inhibited cholycystokinin release is uninhibited which then causes increased pancreatic secretion and consequential pancreatic hypertrophy and hyperplasia (Green and Lyman, 1972; Liddle, Goldfine and Williams, 1984; Liener, 1986). If the secretion of proteases

is considered in terms of a chemical reaction at equilibrium, the removal of the product of the reaction (proteases) drives the reaction to produce more product, as cholecystokinin does for trypsin and other proteases.

It has been postulated that the increased secretion of these endogenous enzymes causes a depletion of amino acids within the animal, thus the dietary protein appears to be of poor nutritional quality. The loss of sulphur amino acids is particularly ac ute since trypsin and chymotrypsin are particularly rich in these (Liener, 1983).

Other more obvious effects are seen in animals which have consumed protease inhibitors. These include reduced food intake, poorer growth and nitrogen retention (Pusztai, 1967; Kakade, Hoffa and Liener, 1973; Liener, 1978; Liener and Kakade, 1980; Pusztai, 1985). The reduced growth and nitrogen retention may be accounted for by the increase in nitrogen which is associated with an increase in digestive enzyme production. This proposal is partially substantiated if the relationship of protein efficiency ratio (PER) with pancreas weight is studied. The PER is negatively correlated with decrease in pancreas weight and is therefore negatively correlated with increased digestive enzyme production (Liener, 1978). PER has been shown to be affected by trypsin inhibitor activity however no correlation can be made between inhibitor activity and PER (Kakade, Simons, Liener and Lambert, 1972, Liener, 1978).

It has also been shown however, that endogenous losses of nitrogen as DNA occurs because of an increased rate of turnover of mucosal cells in the digestive tract when legume protein is consumed (Sandaradura and Bender, 1985). In consequence of the losses of nitrogen by the above mechanisms, availability of nitrogen for body tissue protein synthesis will be reduced.

There is some difficulty in making a comparison between the trypsin inhibitor content and activities from different species of legume because of the variety of methods of determining and expressing these in the literature. Table 1.8.3 summarises data obtained from different plant and literature sources. From the data it is obvious that soya beans and winged beans contain potent trypsin inhibitors which also account for a considerable proportion of the seed protein. It would appear that increased activity is directly related to the proportion of the seed protein (Table 1.8.3) which is the inhibitor thereby indicating that the amount of trypsin inhibitor in the seeds is of greater significance than the activity of the inhibitor. Other workers (Prabhu, Saldanha and Pattabiraman, 1984) have recently shown similar results where soyabean trypsin inhibitor is as potent as that obtained from redwood seed, all others (except red gram) having less than 25% of the activity of these. Significantly *Canavalia ensiformis* had only 5% of the activity of redwood seeds. Other earlier work showed that redwood

Table 1.8.3 Trypsin inhibitors in some legume seeds

SEED	(------trypsin inhibitor----------) (units kg^{-1}*)	(--------g kg^{-1}--------+)		REF.
	seed	seed	protein	
P. tetragonolobus	27.5	28.4	74.9	1
P. sativum	2.0	2.0	8.2	2
P. sativum	2.6	2.7	11.1	3
P. sativum	1.4	1.4	5.7	4
G. max	23.4	24.2	43.3	8
G. max	25.3	26.2	66.5	2
G. max	59.4	61.4	155.8	3
G. max	68.4	70.8	136.1	5
V. faba	2.6	2.7	9.5	3
V. faba	1.5	1.6	5.7	4
L. albus	0-1	0-1	0-2.5	1,2
L. mutabilis	0.41	0.43	2.6	1,2
L. angustifolius	0-1	0-1	0-3.0	1,2
L. luteus	0-1	0-1	0-2.6	1,2
C. tetragonolobus	11.9	12.3	27.4	6
V. sativa	6.5	6.7	24.5	7

* known as TIU, ** known as TI.

Ref. 1: Khor, Tan & Wong, 1982; 2: Hove & King, 1979; 3: Valdebouze, Bergeron, Gaborit & Delort-Laval, 1980; 4: Griffiths, 1984; 5: Kakade, Hoffa & Liener, 1973; 6: Ologhobo & Fetuga, 1984; 7: Kaur & Bhatia, 1984; 8: Charpentier & Lemmel, 1984.

seeds and soya seeds contained the greatest protease inhibitor activity amongst a large number of seeds of tropical origin examined (Sumathi and Pattabiraman, 1976).

The most common method used for the determination of trypsin inhibitor is based on the ability of a plant extract to inhibit the hydrolysis of of a synthetic ester, benzoyl arginine-3-nitroanalide (BAPNA), by trypsin (Erlanger and Kokowski, 1961; Kakade, Simons and Liener, 1969; Kakade, Rackis, McGee and Puski, 1974). The hydrolysis which occurs liberates the yellow coloured 3-nitroaniline which is monitored spectrophotometrically at 405nm and the decrease in production, compared to inhibitor free enzyme, is the degree of inhibition. The results are expressed in different ways but frequently as mg of trypsin inhibited or trypsin inhibitor units (TIU) which can be variously defined. A modification to the original method whereby the released 3-nitroaniline is diazotised and coupled to N-B-sulfatoethyl-2-toluidine prior to detection at 510nm, gives much increased sensitivity (Unger and Braun, 1981).

1.8.2 Haemagglutinins (Lectins)

Recently there has been considerable discussion in the literature regarding the definition of a lectin (Kocourek and Horejsi, 1983). The difficulty in defining, accurately what a lectin is, is due to the wide variety and source of 'lectins'. The term lectin used here is in accord with that which has been accepted by the International Union of Biochemistry (Goldstein, Hughes, Monsigny, Osawa and Sharon, 1980; Dixon, 1981). Lectins are an assortment of proteins and glycoproteins that share the ability to bind stereospecifically and reversibly to carbohydrates. Details of binding sites and specificities of particular lectins for carbohydrates and glycoproteins have recently been summarised (Wu and Herp, 1985). Another important characteristic of a lectin is that it will agglutinate cells and be derived from a non-immune source (Goldstein, Hughes, Monsigny, Osawa and Sharon, 1980). The ability of lectins to agglutinate red blood cells is frequently utilised as a method for their detection and quantification (Goldstein and Hayes, 1978; Valdebouze, Bergeron, Gaborit and Delort-Laval, 1980). Quantification usually means determining the activity (agglutinating ability) of the extracted lectin towards red blood cells. This varies depending on both the source of lectin and blood (Lee, Tan and Liew, 1977; Goldstein and Hayes, 1978; Lis and Sharon, 1981; Grant, More, McKenzie, Stewart and Pustai, 1983). Because of the difficulty in assaying lectins few data exist on the amount of lectin present in legume seeds but rather haemagglutinating activity (HA) is presented.

The foregoing arguments indicate the difficulty in comparing HA of different seeds assayed in different laboratories. A brief summary of HA with content in

Table 1.8.4 Haemagglutinin content and activity of some legume seeds.

SEED	CONCENTRATION IN SEED (mg g^{-1})	HAEMAGGLUTINATING ACTIVITY (HU g^{-1})*	REF.
P. vulgaris	----	140-270	1
P. sativum	----	10-40	2
L. albus	----	0.01	2
L. angustifolius	----	0.005	2
L. luteus	----	0.005	2
G. max	2.5-12.2	160-320	2,3
Lathyrus spp.	1-2	----	4
P. tetragonolobus	6.1- >15	----	5,6
C. ensiformis	4.9-30	----	7,8

* Haemaglutinin Units/g sample (*10e -4) References: 1. Thompson, Rea & Jenkins, 1983; 2. Valdebouze, Bergeron, Gaborit & Delort-Laval, 1980; 3. Pull, Pueppke, Hymowitz & Orf, 1978; 4. Rouge & Pere, 1982; 5. Kortt & Caldwell, 1985; 6. Roy & Singh, 1986; 7. Raychaudhuri & Singh, 1986; Ellis & Belmar, 1985.

seeds is shown in Table 1.8.4. Other data on the relative activities of haemagglutinins from easily accessible legume seeds in the UK have been reviewed (Grant, More, McKenzie, Stewart and Pusztai, 1983). Of the beans tested in that study soya, winged, lima, pinto, aduki beans with pigeon peas, chick peas and blackeyed peas, were ranked amongst the most potent with respect to their HA.

From the literature and the data presented it is not possible to determine whether the proteins in the seeds have a high HA related to weight of protein or if the HA is weaker, but a higher proportion of the protein has HA.

The affinity of lectins is particularly strong for the sugar moieties of glycoproteins and glycolipids (Wu and Herp, 1985). Lectins can be split into two carbohydrate specific groups, those with galactose specificity or those with glucose/mannose specificity (Shannon, 1983).

Lectins in legumes are reported to be associated in various ways with the important ability of legumes to fix nitrogen (Barondes, 1981; Goldstein and Hayes, 1978; Peunans, Stinnisen and Carlier, 1983; Hosselet, Driessche, Van Pouke and Kanarek, 1985). Removal of lectins, genetically, in the growing plant to make it more palatable may affect its nitrogen fixing capability.

Lectins are much larger molecules than protease inhibitors (RAM 36000-265000) and vary widely in composition between leguminous species (Lee, Tan and Liew, 1977; Lis and Sharon, 1981; Grant, More, McKenzie, Stewart and Pusztai, 1983). In some cases the RAM is dependant on the environment because lectins have a tendency to aggregate under certain conditions. Thus concanavalin A, a well studied and defined lectin (Goldstein and Hayes, 1978; Franz, Ziska, Flemming, Horejsi and Ticha, 1985) from *Canavalia ensiformis*, exists as a tetramer at physiological pH (RAM= 102000) but at pH less than 6 its RAM is 51000 (Wang, Cunningham and Eddelman, 1971; Takeo, Fujimoto and Kuwahara, 1983) while the monomeric forms are identical, with RAM of 25500 (Franz, Ziska, Flemming, Horejsi and Ticha, 1985).

There is also considerable variation within species and detailed study shows that, as for trypsin inhibitors, there are a variety of isolectins present within a plant (Riikola and Weber, 1982; Bessler, Kraut, Busing, Muller-Hermes and Peters, 1983; Kortt and Caldwell, 1985). Recently an isolectin of Con A has been identified in jack beans (Raychaudhuri and Singh, 1986).

The voluminous amount of literature concerning lectins is, no doubt, caused primarily by two factors their antiphysiological properties and their potential uses in pharmacology, and in biochemical separation techniques (Lis and Sharon,

1981, 1986; Goldstein and Hayes, 1978; :see also Bog-Hansen and Spengler, 1983; Bog-Hansen and Breborowicz, 1985). An extremely detailed review of the source, variety, and properties of lectins has also been published (Goldstein and Hayes, 1978).

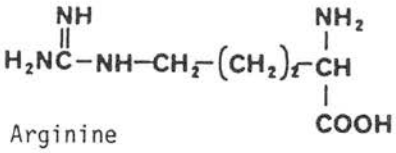
The effect of lectins on animals vary widely depending on their source and on the animal which has consumed them (Liener, 1974; Liener, 1978; Jaffe, 1980; Valdebouze, Bergeron, Gaborit and Delort-Laval, 1980; Lis and Sharon, 1981; Nakata and Kimura, 1986). In some cases lectins such as those from soya bean, produce no adverse effects whereas in others severe growth retardation, the production of liver lesions and, in some cases, death can result after ingestion or injection (Jayne-Williams, 1973; Liener, 1978; Jayne-Williams, 1978; Jaffe, 1980; Richer, Carriere, Blythman and Vidal, 1982). The mechanism of toxicity of lectins is not completely understood although the subject has been the subject of intense study. Some reports (Liener, 1974; Liener, 1978) suggested that the toxicity of lectins was due to a reduction in absorption of nutrients caused by lectins combining with the cell proteins and carbohydrates in the gastrointestinal tract (Jaffe, 1980). However Japanese quail fed raw jack bean and concanavalin A (Con A) died (Jayne-Williams, 1973). Autoclaved jack bean was not lethal but reduced growth. Gnotobiotic quail on the other hand did not succumb to raw jack bean or Con A although growth was depressed. The growth depression may be partially attributable to a reduction in the absorption of nutrients and partially to the effects of other toxic components in jack beans. The obvious conclusion from these experiments was that Con A itself is not lethal but its effect on the gastrointestinal tract of the quail reduced their ability to cope with bacteria, which then caused fatal results.

Recent experiments, primarily with *Phaseolus vulgaris* seeds and their lectins have shown that nutrient absorption is decreased by disruption of intestinal cells, while larger than normal absorption of deleterious compounds is observed (Pusztia, Clarke and King, 1979; Gatehouse, Dewey, Dove, Fenton and Pusztai, 1984; Grant, Greer, McKenzie and Pusztai, 1985). It has also been reported that rats fed Con A, excreted most of it within four days of ingestion (Nikata and Kimura, 1986). These studies also demonstrated that Con A, during transit through the gastrointestinal tract, interfered with brush luminal border membrane and associated enzymes reducing their functional capability with consequential effects on dietary intake.

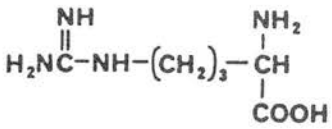
1.8.3 Non-protein amino acids

There are more than 200 non-protein amino acids which have been isolated from plants, the majority of these have been isolated and identified in the last two decades (Fowden, 1981). Legumes have proved to be a major source of these

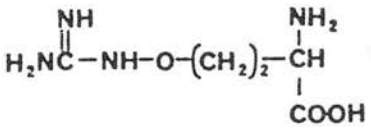
Fig. 1.8.3.1 Analogous structures of some non-protein and protein amino acids



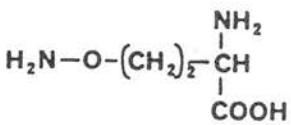
Arginine



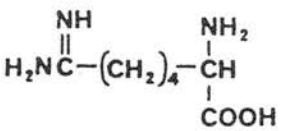
Homoarginine



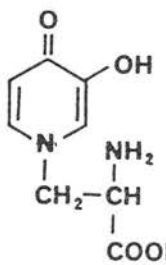
Canavanine



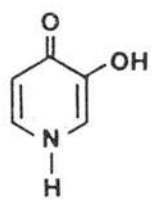
Canaline



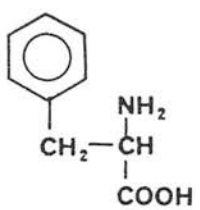
Indospicine



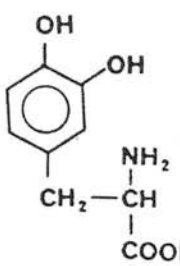
Mimosine



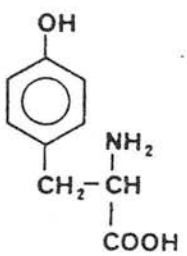
3-hydroxy-4 (1H)-pyridone
(3,4-DHP)



Phenylalanine



3,4-dihydroxyphenylanine (DOPA)



Tyrosine

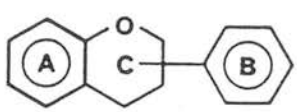
amino acids which are thought to be present as nitrogen storage components and as a method of protection against predation since they tend to be toxic if consumed (Bell, 1972; Bell, 1978; Roy, 1981; Fowden, 1981; Bell, 1981). These unusual amino acids are reported to be present in some legume seeds at concentrations up to 100g kg^{-1} and tend to be analogues of proteinaceous amino acids (Fig.1.8.3.1). The analogous structures of the amino acids (which, in common with protein amino acids, are predominately S diasteriomers) is considered to be one of the reasons why they are toxic. Canavanine [2- amino-4-(guanidinooxy) butanoic acid] and its metabolite, canaline [2-amino-4-(aminooxy) butanoic acid] from *Canavalia* spp. (Birdsong, Alston and Turner, 1960; Rosenthal, 1977; Bell, Lackey and Polhill, 1978) are analogous to arginine and ornithine respectively (Fig. 1.8.3.1). Canavanine is reported (Rosenthal, Janzen and Dahlman, 1977) to occur at levels up to 130g kg^{-1} in the dry seeds of the legume *Dioclea megacarpa* and can account for up to 55% and 96% of the total storage nitrogen and amino acid nitrogen in other seeds (Rosenthal, 1977b).

Mimosine [B-{N-(3-hydroxy-4-pyridone)}-a-amino propanoic acid] is similarly an analogue of DOPA which is a neurotransmitter in animals. Both mimosine and its metabolite, DHP [3-hydroxy-4(1H)-pyridone] are found in *Mimosa* spp. DOPA itself can also be found in plant species such as *Mucuna* spp. while another analogue of arginine, indospicine [2,7-diamino-7-aminoheptanoic acid] is present in *Indigofera spicata* (Bell, 1972; Hegarty and Pound, 1970).

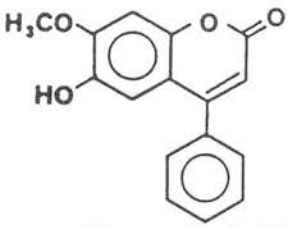
In some cases ingestion of some of these unusual amino acids such as homoarginine, 5-hydroxy-tryptophan and DOPA produce little or no adverse effects on animals although they can inhibit microbial growth. Unusual non proteinaceous amino acids such as homoarginine, when added to rat diets have caused adverse effects on growth, food intake and amino acid balances (Tews and Harper, 1985a; 1985b; 1986a). Homoarginine on the other hand has also been reported to reverse the adverse effects of canavanine on yeasts (Walker, 1955; Bell, 1971).

The toxic effects of the numerous non-protein amino acids are varied and cannot, in some cases, be solely attributed to the amino acid consumed but to the metabolite(s) produced from their degradation. The effects include reduced food intake and growth, alopecia, liver damage, abortions, convulsions, paralysis, skeletal abnormalities as well as other problems including death (Bell, 1972; Roy, 1981). The adverse effects, distribution, biochemistry and chemistry of the amino acids and their derivatives have been sumarised adequately in the literature (Murti and Seshadri, 1967; Bell, 1971; Bell, 1972; Bell, 1981; Fowden, 1981; Roy, 1981; Lea and Miflin, 1980). Various aspects, including analysis, of mimosine, canavanine and their metabolites will be reviewed later in this chapter.

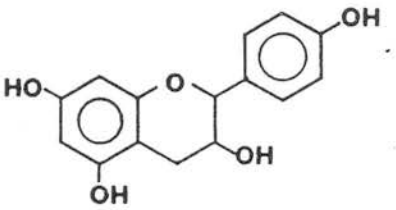
Fig. 1.8.4.1 Representative structures of some of the flavonoid groups



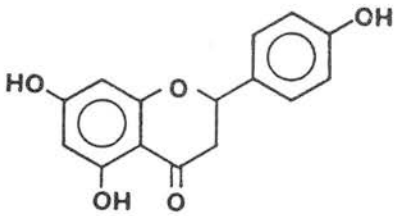
basic structure



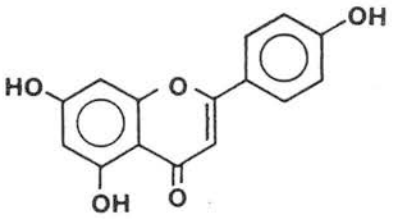
neoflavonoid (dalbergin)



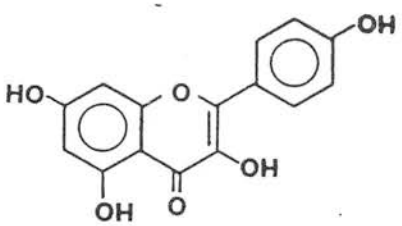
Anthocyanin (pelargonidin)



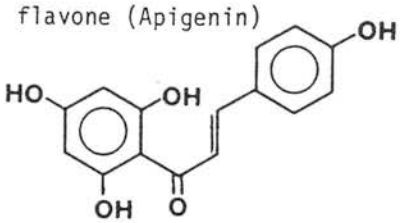
flavanone (naringenin)



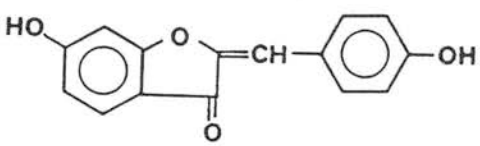
flavone (Apigenin)



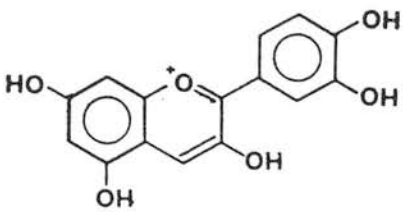
flavonol (Kaempferol)



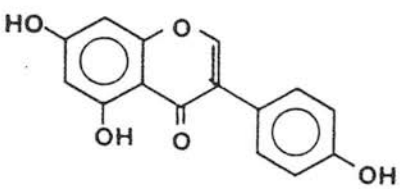
Chalcone (naringenin) chalcone



aurone (hisidol)



Proanthacyanidin (cyanidin)



isoflavone (genestein)

1.8.4 Phenols

Phenols encompass a vast variety of aromatic compounds containing an aromatic nucleus to which is attached one or more hydroxyl groups. Because of the hydrophylicity and reactivity of the hydroxyl groups the phenols, or their extremely large and varied complement of glycosides, are water soluble. They have a strong tendency to hydrogen bond, although other bonding mechanisms have been proposed (Kumar and Singh, 1984), to proteins thus reducing the availability of the protein for animal use (Newman, Newman, El-Negoumy and Aastrup, 1984; Mitaru, Reichert and Blair, 1984). The phenols can legitimately be amino acids, proteins, alkaloids and terpenoids. The compounds which are generally described as phenols, however, include the flavonoids (the largest group), simple monocyclic phenols, phenylpropanoids and phenylquinones. Of the larger phenolics, tannins, lignins and melanins are best known and are frequently described as polyphenolics (Haslam, 1979; Harborne, 1984) and can be found in concentrations up to 60g kg^{-1} in legumes (Marquardt and Ward, 1979; Deshpande, Sathe and Salunkhe, 1984; Kumar and Singh, 1984)

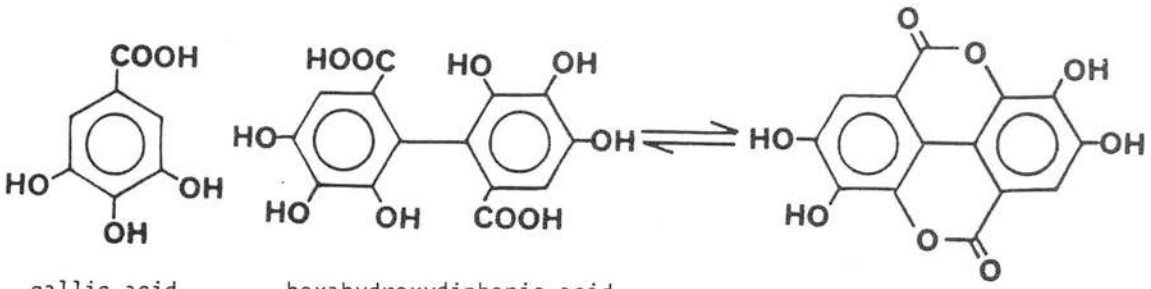
1.8.4.1 Flavonoids

Flavonoids are coloured molecules which are based on a 15 member carbon skeleton (Fig. 1.8.4.1) with a chromane ring to which is attached a second aromatic ring in any of positions 2, 3 or 4. They are phenolic in nature (Fig. 1.8.4.1) and have been reported to be endowed with toxic properties (MacGregor, 1984). Flavonoids comprise ten groups of compounds based on the flavone nucleus (Fig.1.8.4.1) and can occur in plants in their free, unconjugated state, or as glycosides or sulphates (Harborne, 1975; 1984).

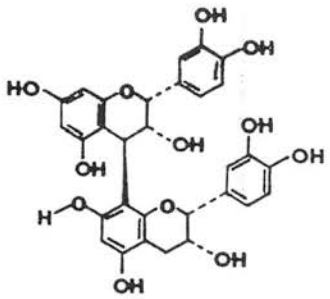
The key structural features in flavonols in making them toxic appear to be the presence of a complete flavone structure with a double bond in positions 2-3 and a hydroxyl group in the 3 position (MacGregor, 1984). Both quercetin and kaempferol have been implicated in gene damage and increased incidence of tumours in mammals (MacGregor, 1984). Other reports have indicated that derivatives and degradation products may be involved in the toxicity of flavonoids (Mclure,1975). Flavonoids may also exert their toxic effects by an anti-vitamin mechanism since *in-vitro* experiments have shown that thiamine activity has been reduced in the presence of quercitin (Yang and Pratt, 1984).

The chemistry, synthesis, biochemistry and significance of flavonoids is extremely interesting, large and complex but outwith the scope of this work. Flavonoids have been comprehensively reviewed by various authors (Harborne, Mabry and Mabry, 1975; Grisbach, 1979; Hahlbrock, 1981; Harborne, 1984). *Leucaena* flavonoids will be discussed later in this work.

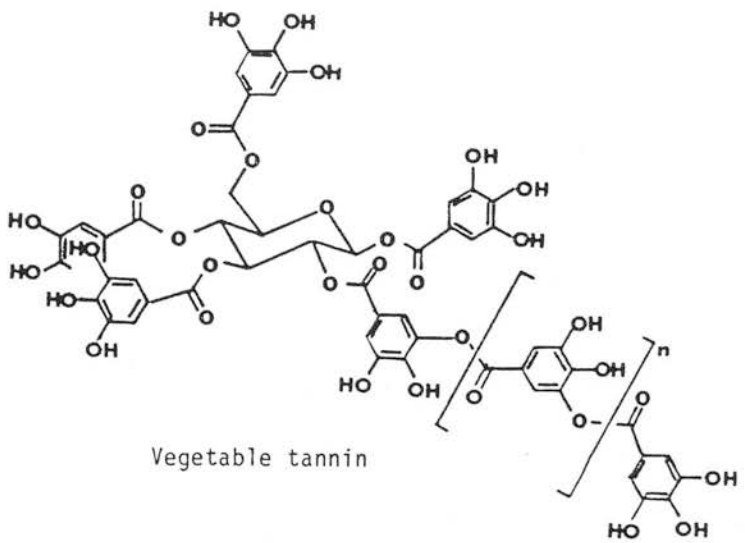
Fig. 1.8.4.2 Structures of some polyphenolics



gallic acid hexahydroxydiphenic acid ellagic acid



Procyanidin B-2



Vegetable tannin

1.8.4.2 Tannins, other phenols and polyphenols

This is another complex group of compounds which are widely distributed particularly in dicotyledons (McLeod, 1974). They contain a large number of hydroxy groups, are water soluble and hydrogen bond to proteins. The hydrogen bonding properties are pH dependent (Oh, Hoff, Haff, 1985). This hydrogen bonding property has been used to estimate the amount of tannins present in foodstuffs (Bressani, Elias, Wolzak, Hagerman and Butler, 1983).

Tannins have been defined as water soluble phenolic compounds which have RAM between 500-3000, give phenolic reactions and have the ability to precipitate alkaloids and proteins (Haslam, 1981). There are two types of tannins, the condensed tannins which are condensation oligomers and polymers of flavonoids (Fig. 1.8.4.1; Mcleod, 1974; Haslam, 1975; Haslam,1979; Kumar and Singh, 1984) and found mainly in forage legumes. The other type are referred to as hydrolysable tannins because of their susceptibility to hydrolysis. This ability to undergo hydrolysis is due to the polyester type of bonding between the constituent phenolic carboxylic acids and the central carbohydrate units (Mcleod, 1974; Haslam, 1981; Dabrowski and Sosulski, 1984; Sosulski and Dabrowski, 1984). Hydrolysable and condensed tannins have been detected in *Leucaena* leaf meal using HPLC techniques (D. Kufidis, unpublished work).

Figure 1.8.4.2 shows the structures of some typical polyphenolics. Attention should be drawn to the two polyphenols, gallic acid and ellagic acid which form the basis of the different types of hydrolysable tannins called gallotannins and ellagitannins. These acids are the usual hydrolysis products (Haslam, 1981). The large variety and complexity of polyphenols and tannins in plants combined with their susceptibility to oxidation, make their quantitation and identification difficult. The availability of highly resolving chromatographic techniques allows elucidation of these phenolic components in plants (Kufidis, 1984, personal communication; Harborne, 1984; Dabrowski and Sosulski, 1984a).

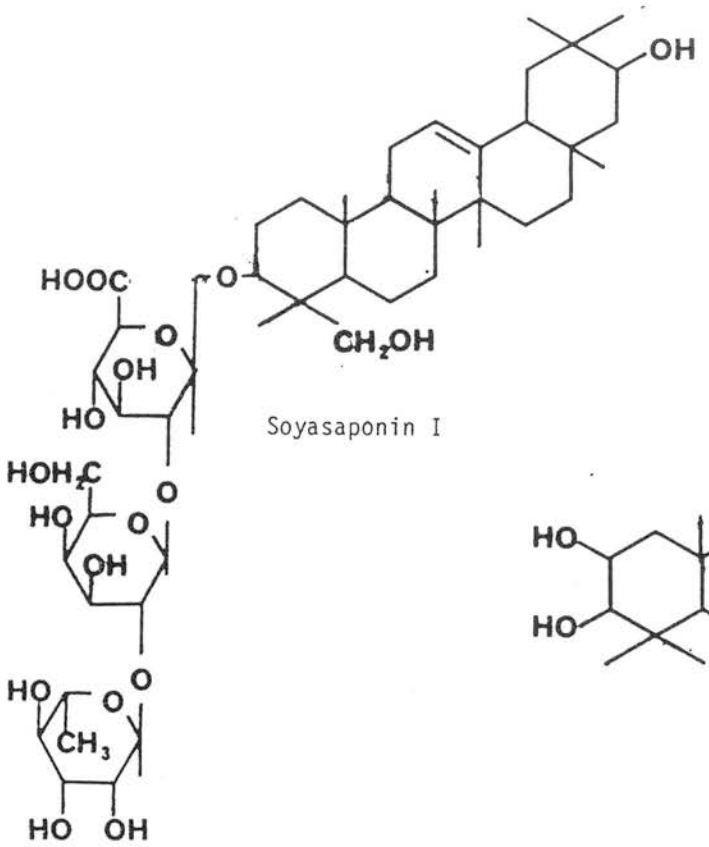
Conventional methods of analysis of tannins haveutilised the phenolic component of these molecules in such reactions as those with iron (III) to produce coloured complexes. Such methods are highly dependent on the type of phenolic compounds present in the plants used. Not surprisingly such non specific methods give highly variable results (Banda and Vohra, 1983; Bressani, Elias, Wolzak, Hagerman and Butler, 1983; Harborne, 1984) as with the estimation of tannins in *Leucaena* (D'Mello and Fraser, 1981). Microbiological and dye binding techniques have also been developed to measure tannins (Ford and Hewitt, 1979a). Because of the variation in methodology of determination of tannin in plant material, values vary considerably for the same sample due to the different compounds detected using the different methods (D'Mello and Fraser, 1981).

The nutritional implications of tannins and their occurrence has been recently reviewed (Reddy, Pierson, Sathe and Salunkhe, 1985). Polyphenols and tannins are reported to exert their antinutritional effects by binding to dietary and digestion proteins (Tamir and Alumot, 1969; Mcleod, 1974; Neucere, Jacks and Sumrell, 1978; Mitaru, Reichert and Blair, 1984). The reduced digestibility of the ingested protein may therefore be due to enzyme deactivation and/or reduction in the susceptibility of the ingested protein by complexing with tannins (Reddy, Pierson, Sathe and Salunkhe, 1985). The degree of binding of the protein by tannin is dependant on the type of tannin in the plant and also on the pH of the media (Jones and Mangan, 1977, Mitaru, Reichert and Blair, 1984). It has been observed that variation in the pH (Jones and Mangan, 1977) of tannin solutions in the presence proteins has varied the amount of bound protein as measured by protein solubility. Addition of hydrophilic polymers such as polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) have also been shown to have considerable effect on protein solubility in the presence of tannins (Tamir and Alumot, 1966; Jones and Mangan, 1977). This effect is caused by PVP and PEG substituting for the protein in the protein tannin complex, forming a soluble PVP/PEG- tannin complex thus releasing the protein. Another mechanism by which tannins are thought to assert their antinutritional effects is by reducing the available methionine in the diet by utilising it as a methyl donor. Supplementation of high-tannin bean diets with methionine, fed to rats, increased the nutritional value of the protein and was postulated to aid in the detoxification of the tannins (Bressani, Elias, Wolzak, Hagerman and Butler, 1983). Earlier work with chicks fed tannin diets and supplemented with methyl donors however did not alleviate the toxic properties of tannic acid (Vohra, Kratzer and Joslyn, 1966).

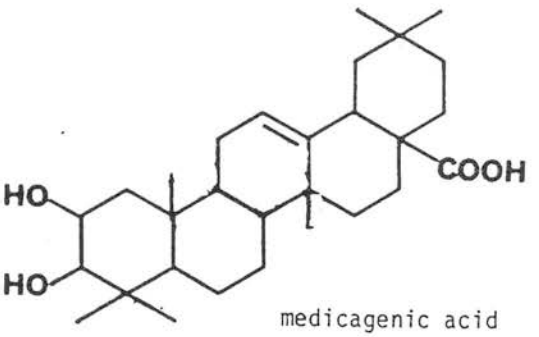
Enzymes inhibited by tannins have been shown to include proteases, amylases and lipases (Tamir and Alumot, 1969; Griffiths and Moseley, 1980). With the inhibition of these mammalian digestive enzymes by tannins, associated with the complexation of tannins with plant proteins (Neucere, Jacks and Sumrell, 1978), the reduction in metabolisable energy (ME) and utilisation of protein and amino acids, reported by various workers, is to be expected when animals are fed diets containing tannins (Marquardt and Ward, 1979; Hewitt and Ford, 1982; Mitaru, Reichert and Blair, 1983; Newman, Newman, El-Negoumy and Aastrup, 1984; Mitaru, Reichert and Blair, 1984).

The adverse effects of tannins on animals has been alleviated to varying degrees by different treatments such as heat, (Marquardt and Ward, 1979; Savage, Smith and Briggs, 1980; Tan, Wong and de Lumen, 1984) water treatment, (Mitaru, Reichert and Blair, 1983, 1984a; Teeter, Sarani, Smith and Hibberd, 1986) PVP and PEG supplementation of diets (Rayudu, Kadirvel, Vohra and Kratzer, 1970;

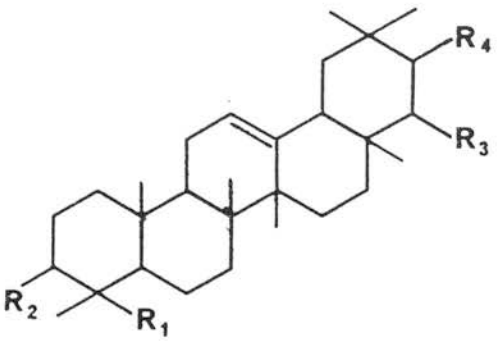
Fig. 1.8.5.1 Structures of some common saponins and sapogenins



Soyasaponin I

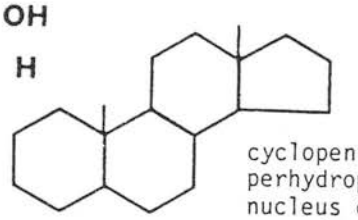


medicagenic acid



triterpenoid nucleus

	R_1	R_2	R_3	R_4
SOYASAPOGENOL A	CH_2OH	OH	OH	OH
SOYASAPOGENOL B	CH_2OH	OH	OH	H



cyclopentane perhydrophenanthrene nucleus of steroid saponins

Savage, Smith and Briggs, 1980; Hewitt and Ford, 1982; Barry, 1983; Kumar and Singh, 1984). Other chemical treatments using ammonia, iron salts and alkalis have also been used with varying degrees of success in reducing tannin content and their deleterious effects (Wah, Sharma and Jackson, 1977; Price, Butler, Rogler and Featherston, 1979; Ford and Hewitt, 1979b; 1979c).

Treatment by heat is considered to be effective in reducing tannin effects by causing polymerisation or oxidation of the tannins. Steric hindrance of the polymerised tannins prevent their reaction with proteins (Jones and Mangan, 1977; Kumar and Singh, 1984). Tannins in diets not only have antinutritional and toxic effects but can also have beneficial effects when consumed by ruminants. The anti-foaming properties associated with tannins means that they tend to reduce foaming in the rumen (bloat) caused by compounds such as saponins (Mcleod, 1974; Kumar and Singh, 1984).

1.8.5 Saponins

Saponins (Fig. 1.8.5.1) are the glycosides of sapogenins which are either steroids or triterpenes (Fig. 1.8.5.1). They are commonly found in legumes (Bell, 1978) in concentrations up to 56g kg^{-1} in the seeds (Oakenfull, 1981; Fenwick and Oakenfull, 1983). They are characterised by their bitter taste, foaming tendency in aqueous solution and their ability to haemolyse red blood cells due to their surface active properties. Heamolysis of blood has been used to quantify saponins but such methods have been superceded in recent years by more descriminative chromatographic techniques in which other more specific and sensitive detection methods are employed (Basu and Rastogi; 1967; Brawn, Lindner, Miller and Telling, 1981; Curl, Price and Fenwick, 1985; Ireland and Dziedzic, 1985; Kitagawa, Yoshikawa, Hayashi and Tanayama, 1984a; 1984b). The variation in analysis methods may account for the large variation in concentrations (4.6-56g kg-1) reported for the saponin content of soya (Ireland, Dziedzic and Kearsley, 1986).

The source and variety of triterpenoid saponins have been thoroughly reviewed (Basu and Rastogi, 1967). Other reviews of source, physiological effects, chemistry and biochemistry of triterpenoid and steroid saponins have also been published (Birk, 1969; Cheeke, 1976; Birk and Peri, 1980; Oakenfull, 1981).

There is considerable variation in the composition of saponins, both from different plant species and within a plant species. Both the sapogenin nucleus and the substituent carbohydrate group are responsible for the variation in structure (Birk, 1969; Blunden, Carabot and Jewers, 1980; Birk and Peri, 1981; Fenwick and Oakenfull, 1981; 1983). Although variation exists between saponins in different species, the various species can also contain some identical saponins

(Blunden, Carabot and Jewers, 1980). The major saponin (soyasaponin I; Fig. 1.8.5.1) in soya has recently been elegantly isolated, quantified and identified in *Pisum sativum* L. (Price and Fenwick, 1984; Curl, Price and Fenwick, 1985). This saponin has also been identified in other legume seeds with the exception of jackbeans (Price, Curl and Fenwick, 1986). However, as with protease inhibitors and lectins, saponins from the same source are reported to elicit differing responses in animals which have consumed them while the quantitative effects of the different saponins also varies (Birk, 1969; Nonaka, 1986).

One of the major roles of saponins in plants has been postulated to be as inhibitors of predation because of their deleterious effects when consumed. Their bitter taste also tends to reduce their platability (Appelbaum, Marco and Birk, 1969; Acamovic; personal observation). Saponins have also been shown to inhibit fungal growth on plants (Holden, 1980; Deacon and Mitchell, 1985; Nonaka, 1986). The association of saponins with plants, fungi and animals is deleterious due to the destabilisation of the cell walls because of their surface active properties.

Saponins have been reported to alter cell wall permeability and therefore to exert some toxic effects when ingested but extreme toxicity when injected (Basu and Rastogi, 1967; Johnson, Gee, Price, Curl and Fenwick, 1986). In a similar fashion to lectins, saponins have been shown to interact with intestinal brush border membranes thereby increasing the permeability of the mucosal cells (Nakata and Kimura, 1985; Johnson, Gee, Price, Curl, Fenwick, 1986). As a result these cells lose their functional capability and become, in a similar fashion to the effects of lectins, permeable to normally innocuous substances.

The ingestion of saponins has been shown to reduce blood cholesterol and lipid levels in rats, chicks pigs and monkeys (Malinow, McGlaughlin, Kohler and Livingston, 1977; Oakenfull, Fenwick, Hood, Topping, Illman and Storer, 1979; Pathirana, Gibney and Taylor, 1981; Malinow, Baranda, Pirofsky, Craig and McGlaughlin, 1982) although the greatest effect was for chicks fed high cholesterol diets supplemented with saponins. Highly significant differences in blood cholesterol levels have been observed when chicks were fed diets containing leaf protein concentrate containing saponins (Ueda, Ohshima, Kamada, 1986). Rabbits fed diets containing saponins, on the other hand, did not show significantly lower levels of cholesterol in their serum (Pathirana, Gibney and Taylor, 1981). There is considerable discussion in the literature regarding the effect of saponins and/or fibre (polymeric carbohydrates) on blood cholesterol and lipid levels (Cheeke, 1976; Oakenfull, Fenwick, Hood, Topping, Illman and Storer, 1979; Pathirana, Gibney and Taylor, 1981). From the polemics in the literature (Gibney, Pathirana and Smith, 1982; Oakenfull and Topping, 1983) it

appears that saponins reduce cholesterol in the blood of hypercholesterolaemic subjects but the connection between fibre and saponin and their combined effect on cholesterol levels in the blood is tenuous.

The evidence is quite clear that incorporation of saponins in diets causes increased excretion of bile acids and lipids (Oakenfull, Fenwick, Hood, Topping, Illman and Storer, 1979; Potter, Illman, Calvert, Oakenfull and Topping, 1980; Pathirana, Gibney and Taylor, 1981) although the reasons for this have not yet been fully elucidated (Cheeke, 1976; Oakenfull and Fenwick, 1978; Calvert and Yeates, 1982). Some very recent work has demonstrated that large micelles between saponins and bile acids are formed (Oakenfull, 1986) and thus the acids cannot be absorbed through the small intestine but pass through the animal (Oakenfull and Sidhu, 1983; Sidhu and Oakenfull, 1986). This effect combined with the formation of complexes between dietary cholesterol and saponins (Malinow, McGlaughlin, Stafford, Livingston, Kohler and Cheeke, 1977) is likely to cause reductions in serum cholesterol levels. Loss of endogenous cholesterol may also occur due to the increased permeability of the intestinal mucosa (Johnson, Gee, Price, Curl and Fenwick, 1986). There is considerable evidence that diets which include saponins reduce growth rate, food intake and efficiency of food conversion (Cheeke, Pedersen and England, 1978, Whitehead, McNab and Griffin, 1981; Livingston, Knuckles, Teuber, Hesterman and Tsai, 1984) although turkeys fed low levels of saponins did not respond adversely (Dziuk, Duke, Buck and Yani, 1985). Experiments in which inclusion of saponins in the diets caused reduced food intake and where lipid and cholesterol levels in tissue and/or serum were lower than for control diets (Morgan, Heald, Brooks, Tee and Green, 1972; Whitehead, McNab and Griffin, 1981) confound the observations. The reduced food intake of isoenergetic and isolipidic diets, and hence reduced energy and lipid intakes, will reduce lipid and cholesterol levels in the animals (Whitehead, McNab and Griffin, 1981).

Eggs obtained from saponin fed hens have been shown to have reduced cholesterol concentrations (Godfrey, Luttinger, Taylor and Sanhueza, 1976; Whitehead, McNab and Griffin, 1981). Other effects caused by the consumption of saponins include the development of lesions in the intestines and reduced reproductive capability (Cheeke, 1976; Feher and Bosca, 1984).

1.8.6 Carbohydrates

Carbohydrates can be defined as polyhydroxy aldehydes or ketones having RAM ranging from less than 100 to considerably larger than 1 million. Because of the large variety of carbohydrates available, an extensive literature exists on their synthesis, function, metabolism and uses (Stumpf and Conn, 1980; Duffus and Duffus, 1984; Lewis, 1984; Vliegenthart, Kamerling and Veldink, 1984).

Information on the composition of legume carbohydrates, specifically, is also abundant (Bailey, 1971; Arora, 1983; Reddy, Pierson, Sathe and Salunkhe, 1984) Large molecular weight carbohydrates and their nutritional implications has also recently been thoroughly reviewed (Walter, 1985).

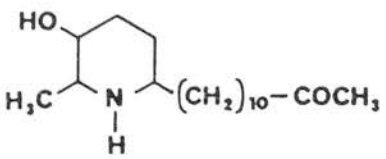
Most of the commercially important legume seeds contain starch as their major polysaccharide however there is considerable variation in the composition and content of starches from various species of legume (Norton, Bliss and Bressani, 1985). The starch content of the majority of important legumes varies between 300 to about 600 g kg^{-1} (Reddy, Pierson, Sathe and Salunkhe, 1984). A noteable exception is guar in which most of the polysaccharide is present as the mucilinageous guar gum which has no nutritional value (Verma, 1977) and may even be a deleterious component of guar (Ray, Pubols and McGinnis, 1982; Struthers, 1986). It is of note that guar gum has a large variety of uses (Arora, 1983). Other important legumes in which starch is a minor component of the carbohydrate in the seeds are soya bean, lupin and winged bean (Reddy, Pierson, Sathe and Salunkhe, 1984).

A number of monosaccharides have been found in legumes but the most common of these, either as the free carbohydrate or in conjugated form, is glucose (Arora, 1983). Of the oligosaccharides found in legumes sucrose, raffinose, stachyose and verbascose are the most common (Newton and Hill, 1983; Lourdes, Bianchi, Silva and Braga, 1984, Bliss, Norton and Bressani, 1985) and are attributed as the flatulence causative factors in legumes (Fleming, 1981; Jood, Mehta, Singh and Bhat, 1985). These carbohydrates all contain a-(1->6) galactosidic bonds which make them indigestible allowing them to pass into the lower gut where they undergo anaerobic fermentation by bacteria (Jood, Mehta, Singh and Bhat, 1985).

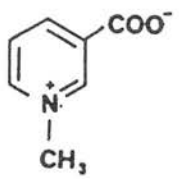
Legume polysaccharides include structural carbohydrates such as celluloses, hemicelluloses together with lignin (Reddy, Pierson, Sathe and Salunkhe, 1984) which are not digested in non-ruminants and are determined as fibre in the plant. As can be seen (Table 1.7.1) legume seeds have a much lower fibre content than the foliar material. Pectic polysaccharides are the other polysaccharides present in legumes (Champ, Brillouet and Rouau, 1986). These carbohydrates can be generally characterised into three groups; the pectins which are polymers of galacturonic acid, the galactans and arabans which are heteropolymers of galactose and arabinose respectively (Dey, 1980, Arora, 1983; Duffus and Duffus, 1984).

Galactomannans are of particular interest in legume seeds. They are reserve polysaccharides composed of linear chains of (1-->4)-linked B-D-mannopyranosyl residues having single stubs of a-D-galactopyranosyl groups

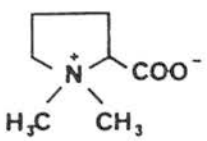
Fig. 1.8.7.1 Structures of some alkaloids found in the Leguminoseae



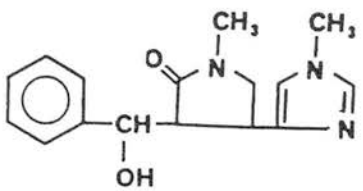
Cassine (piperidine)



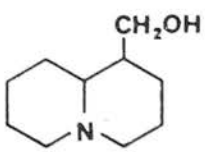
Trigonelline (pyridine)



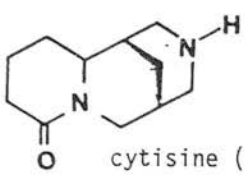
Stachydrine (pyrrolidine)



Cynometrine (imidazole)



(-)-Lupinine
(quinolizidine)



cytisine (quinolizidine)

1 - methylene pyrrolizidine
(pyrrolizidine)

joined by (1-->6)-linkages along the chain. The chemistry, biochemistry and occurrence of these have been thoroughly reviewed (Dea and Morrison, 1975; Dey, 1978; Dey, 1980). These pectic carbohydrates have been shown to be associated with cell walls (Hesselman, 1983; de Silva, Hesselman and Aman, 1983; Parker, 1984; Crawshaw and Ried, 1984; Carre, Brillouet and Thibault, 1985) and are at best of no nutrititive value for monogastric animals and simply act as diluents in diets (Hove and King, 1979; Grammer, McGinnis and Pubols, 1982; Carre and Leclercq, 1985). Numerous reports have, however, shown that the ingestion of such carbohydrates reduce growth rate and food intake (Vohra, Shariff and Kratzer, 1979; Ray, Pubols and McGinnis, 1982; Delorme and Gordon, 1984; Carre and Leclercq, 1985). Pectic polysaccharides have been implicated in reduction of cholesterol levels and lipids in man and animals and are associated with increased lipid excretion and rachitogenic effects in poultry (Gee, Blackburn and Johnson, 1983; Grammer, McGinnis and Pubols, 1985; McNaughton, Morrison, Huhner, Ernest, Ellis and Howell, 1985; Walter, 1985). Increased lipid excretion in rats due to increased ingestion of polymeric carbohydrates is, at least in part, caused by increased excretion of bile acids (Walter, 1985; Walter, Eastwood, Brydon and Elton, 1986). It seems probable therefore that dietary AME will appear to be reduced because of the ingestion of undigestible carbohydrate, not because of the lack of energy from the carbohydrate *per se*, but because of the increased excretion of lipid as bile acids. The effect of AME reduction is similar to that reported for the ingestion of saponins and cholesterol in chicks (Ueda, Ohshima and Kamada, 1986).

Work with rats (Sharma, 1984) has also shown that serum low density lipids are reduced relative to controls, although cholesterol levels were not, when hypercholesterolaemic diets containing various gums were ingested. In common with this work, that of other workers (Chang, Ethen, Harrold and Brown, 1986) where rats were fed various beans, serum cholesterol levels were not significantly affected.

1.8.7 Alkaloids

Alkaloids are nitrogen containing compounds that are usually heterocyclic and tend to be basic. The pKa of a typical lupin alkaloid has been reported to be 9 (Leonard, 1960). They are a heterogeneous set of compounds, comprising about 6000 in number although they are not all present in legumes (Harborne, 1984). They include quinolizidine, indolizidine, pyrrolizidine, isoquinoline, pyrrolidine, piperidine, pyridine, indole, tropane, imidazole and amaryllidaceae alkaloids. A structural representative of some of these alkaloids are shown in Fig. 1.8.7.1. These and other alkaloids are the subjects of recent reviews by various authors (see: Nat. Prod. Rep., 1984 & 1985; Wink, 1984). The occurrence, chemistry and

biochemistry of the various alkaloids have also been the subject of regular reviews since 1950 (The Alkaloids). The structures of a wide variety of alkaloids found in the Caesalpinioideae and Mimosoideae (Smolenski and Kinghorn, 1981), and in the Papilionoideae (Kinghorn and Smolenski, 1981) have been compiled.

The precursors of alkaloids in nature invariably include an amino acid; the production of sparteine, a quinolizidine alkaloid of lupins, is synthesised from lysine (Wink, 1984; Haslam, 1985). It is therefore not surprising that low alkaloid varieties are reported to have a higher lysine content than high alkaloid varieties (Hill, 1977; Green and Oram, 1983). Because of the widely varying physiological responses to the large array of alkaloids, they have come under intense study particularly with respect to their potential pharmacological uses (Waller and Dermer, 1981). Their presence in plants is considered to be part of the plant defence mechanism not only because of their toxicity but also because of the bitter taste associated with them (Harborne, 1984; 1986; Godfrey, Mercy, Emms and Payne, 1985; Wink and Witte, 1985). The newer 'sweeter' varieties of legumes containing alkaloids such as lupins, although they do not have much taste initially, leave an unpleasant effect in the mouth after ingestion (Acamovic, unpublished observation) and therefore do not encourage further consumption. Bitter (high alkaloid) varieties of lupins fed to chicks reduced feed intake and this reduction has been attributed to the reduced palatibility of the diets caused by the alkaloids (Guillaume, Chenieux and Rideau, 1979).

Lupin alkaloids have varying degrees of toxicity and are of considerable interest and will be discussed in more detail later.

1.9 *LEUCAENA LEUCOCEPHALA*

Leucaena leucocephala (*leucaena*) is a tropical leguminous tree or bush which has received considerable attention over the years. The evidence for such a statement is found in the large bibliography containing more than 2000 references on the subject (Oakes, 1982 & 1983). *Leucaena* originated in Mexico and Central America but is now well distributed throughout the world (Gray, 1968; NAS, 1984). Because of its wide distribution it is known by many names (Skerman, 1977). There are ten species of *leucaena* and about 800 varieties (Brewbaker, 1982) found in various environments and which have widely varying characteristics (Gray, 1968; Oakes, 1968; Blom, 1980). They vary from small bushes of about 5m in height to large trees of about 20m (NAS, 1984) and are reported to be able to fix dinitrogen at rates between 100 and 500 kg ha^{-1} $annum^{-1}$ (Blom, 1980; NAS, 1984). Growth and regrowth of *leucaena* is very rapid, under good conditions the plant can grow up to 6m in twelve months and produce up to about 130 tonnes fresh forage ha^{-1} yr^{-1}. however this rate of production was achieved under irrigation. Production at this rate is comparable or

better than that for Lucerne (Blom, 1980; NAS, 1984). Best growth occurs below an altitude of about 500m between latitudes of 30 degs N and S of the equator where rainfall is between 1000-3000 mm annum^{-1}. There is considerable variation in optimal growth conditions depending on the area of the world in which *leucaena* is grown, it has been reported to be grown at altitudes greater than 1000m in Indonesia (Oakes, 1968). *Leucaena* grows well on alkaline soils of low fertility and is susceptible to P and S deficiencies (Gray, 1968; Blom, 1980; NAS, 1984). Unusually for tropical plants the *rhizobia* necessary for *leucaena* establishment are the slow growing alkaline producing type found in the tropics (Hill, 1971; Halliday, 1981; Jones and Bray, 1983) rather than the fast growing acid producing type, more commonly found in tropical legumes.

1.9.1 Uses

Leucaena can be grown as a forage crop for ruminants although if used for this purpose precautions must be taken in order to avoid the various toxic effects which can include death, and have been attributed to mimosine. Further details on the toxic and nutritional aspects of *leucaena* will be presented later in this section.

Other uses include the production of wood, both for fuel (Blom, 1980; Van Den Beldt and Brewbaker, 1980; Othman and Prine, 1984) and for furniture, pulping and structural supports (Blom, 1980; NAS, 1984). *Leucaena* improves the soil condition where it grows both because of dinitrogen fixation and provision of organic fertiliser from the foliage, soil stability is increased thus reducing erosion because of its rapid growth and its deep root system. It can also act as a shade or companion crop (Pound Santana and Ruiz, 1980; Benge, 1981a; Das and Reddy, 1982).

1.9.2 Feedingstuff

As mentioned earlier *leucaena* has potential as a nutritious forage for ruminants or, after drying, as a supplement (as *leucaena* leaf meal; LLM) to non ruminant diets. The attributes and disadvantages of *leucaena* as a feedstuff is the subject of a number of reviews (Oakes, 1968; D'Mello and Taplin, 1978, Jones, 1979; ter Meulen, Struck, Schulke and El Harith, 1979).

Reports on the effects of ingestion of *leucaena* on ruminants indicate that responses of the animals are variable. In some cases no adverse effects have been reported when *leucaena* is consumed (Thomas and Addy, 1977; Jones, 1981; Jones and Megarrity, 1983) but more frequently, deleterious effects are reported (Hegarty, Schinkel and Court, 1964, Holmes, 1981; Jones and Megarrity, 1983; Jones and Hegarty, 1984). The variation of response of animals has been

attributed to a number of possible causes which include species of animal and *leucaena* used, the mimosine content, the rate of introduction to *leucaena* and, more recently, the presence or absence of rumen microflora which are capable of metabolising mimosine and 3,4-DHP (Hegarty, Court and Schinkel, 1964; Jones and Megarrity, 1983).

Growing pigs, fed low mimosine and low tannin LLM diets (200g kg^{-1}) have been shown to perform better than pigs fed isoenergetic and equinitrogenous control diets (Gonzalez Vargas and Wyllie, 1982a). Digestibility coefficients for LLM diets for pigs were found to be lower than for control fed pigs; the apparent metabolisable energy (AME) of the LLM used was determined to be 5.35MJ kg^{-1} (Gonzales Vargas and Wyllie, 1982b).

The AME of LLM for young chicks has been determined to be between about 2.2 to 2.7MJ kg^{-1} (D'Mello and Thomas, 1978, D'Mello and Acamovic, 1981) which is about the same as, or less than, lucerne but about half that of young dried grass (De Groote, 1974). The grass, lucerne and LLM had fibre contents of 217, 315 and 84g kg^{-1} respectively yet it is of note that the AME of LLM for poultry is lower than might be expected if fibre was the only factor affecting AME (De Groote, 1974; Herstad, 1975; D'Mello and Taplin, 1978; D'Mello and Fraser, 1981) although the fibre content of LLM is considerably lower. The low AME of LLM may be due to a variety of factors including the presence of tannins, saponins, unusual carbohydrates, and the type of lipid present in LLM (see previous sections and Wiseman, Cole, Perry, Vernon and Cooke, 1986).

Interestingly the AME of *leucaena* for sheep (7.1-8.2 MJ kg^{-1}) is very similar to that of lucerne (8.4 MJ kg^{-1}) which may indicate a similar degree of lignification between the two (McDowell, Conrad, Thomas and Harris, 1974; Minson, 1982; Wong and Devendra, 1983.)

Leucaena meal, substituted for lucerne, in broiler diets reduced growth rate of the broilers and caused lethargy (ter Muelen, Pucher, Szyszka and El-Harith, 1984). A possible explanation for the highly significant reduction in growth may be that the *leucaena* meal used contained a high proportion of seed (64%) which is likely to have contained other toxic components such as trypsin inhibitors and haemagglutinins. It is well documented, however, that inclusion of LLM in poultry diets produces growth depressions (Ross and Springhall, 1963; Gloria, Gerpacio, Aglibut and Castillo, 1966; Labadan, 1969; D'Mello and Acamovic, 1981 Hegde, Ross and Brewbaker, 1983). In some cases dietary inclusion of LLM at levels of 50g kg^{-1} in isoenergetic diets has produced growth depressions (D'Mello and Thomas, 1978) whereas in others inclusion at 150g kg^{-1} has had no adverse effect (D'Mello and Acamovic, 1982).

Table 1.9.2.1 Proximate composition of leucaena leaf meal. (expressed per 1000g dry matter)

Source	Ash (g)	CP[@] (g)	EE (g)	Fibre (g)	GE (MJ)	Ref.
Malawi	232.9	297.5	47.6	-	-	1
Malawi	110.5	259.0	26.4	118.8[+]	20.1	2
Nigeria	82.0	313.0	19.0	128.0[*]	-	3
Nigeria	90.0	268.0	29.0	144.0[*]	-	3
India	55.4	299.8	35.6	167.2[*]	-	4
Thailand	97.8	224.4	39.8	123.6[£]	-	5
Mexico	94.6	300.0	14.5	168.0[?]	19.3	6
MEAN	109.0	280.2	30.3	141.7	19.7	
+ sem	8.18	4.47	1.66	3.66	-	
-						

References 1: D'Mello & Thomas, 1978; 2: D'Mello & Taplin, 1978; 3: Adeneye, 1979; 4: Sobale, Kharat, Prasad, Joshi, Rangnekar & Deshmukh, 1978; 5: D'Mello & Fraser, 1981; 6: Alvarez, Wilson & Preston, 1978.

[+]: Trichloro acetic acid Fibre; [*]: Crude fibre; [£]: Modified acid detergent fibre; [?] : Unknown method of fibre determination; [@]: nitrogen* 6.25.

Various methods to reduce the deleterious effects of *leucaena* on poultry have been attempted. These include washing, supplementation with metal salts, supplementation with compounds which structurally resemble mimosine such as tyrosine, niacin and pyridoxal phosphate, and fermentation with rumen liquor, prior to feeding (Ross and Springhall, 1963; Castillo, Aglibut, Gerpacio, Gloria, Gatapia and Resurreccion, 1964; Gloria, Gerpacio, Aglibut and Castillo, 1966; Labadan, 1969). All of the foregoing treatments of *leucaena* were designed to reduce the adverse effects on growth of poultry, attributed to mimosine. Washing *leucaena* with water has been shown to reduce the mimosine content by about 50% however it seems reasonable that water soluble nutrients would be leached out of the *leucaena*; no information was given as to the nutrient content of *leucaena* prior to, or after washing. Treatment of *leucaena* with rumen liqour has been shown to have little effect on mimosine content in some cases while in others, substantial losses occurred (Labadan, 1969; Shiroma and Akashi, 1976). The difference in effect may have been due to the absence and presence, respectively of mimosine degrading microflora in the rumen liquor used (Jones, 1981). No information was presented on the effect of such treatment on the nutrient content of the *leucaena*. In all cases, with the exception of the addition of mimosine analogues, contrary to work with rats (Lin, Lin and Tung, 1964), beneficial but not complete responses in growth were elicited. In one report (Ross and Springhall, 1963) it was suggested that Fe(II)-phosphate complexes in the diet may have moderated the beneficial effects of ferrous sulphate supplementation.

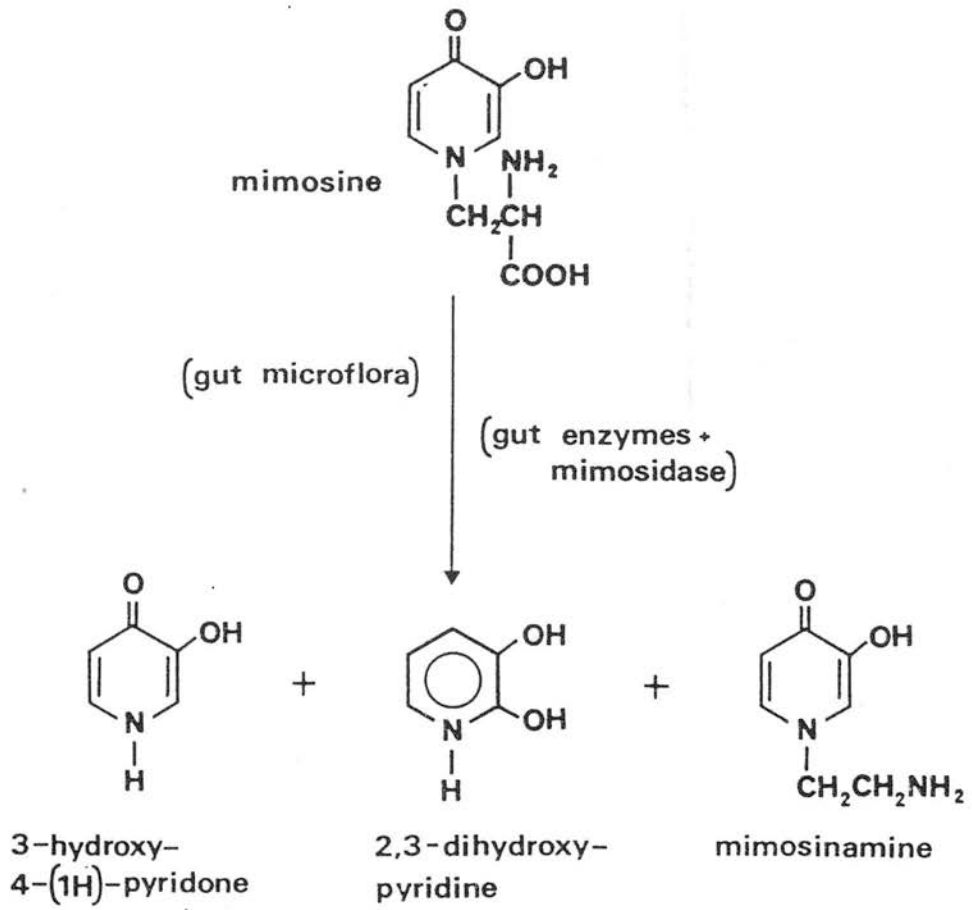
The deleterious effects of LLM on growth of chicks have also been ameliorated as protein levels of the diets were increased (Hathcock, Labadan and Mateo, 1975). Chicks, in these experiments, fed diets with identical levels of LLM but different protein concentrations grew better at higher protein levels. The growth of chicks fed their respective control diets (ie. isonitrogenous with the LLM diets) was always higher than their corresponding LLM diets even when supplementation was lowest at 129g kg^{-1}.

1.9.2.1 Composition of Leucaena

Leucaena is a relatively nutritious feedstuff. Its proximate analysis is similar to that for lucerne although the fibre content of lucerne is higher than that for *leucaena* (D'Mello and Taplin, 1978). The proximate analysis of *leucaena* from different areas of the world and harvested by different methods is shown in Table 1.9.2.1.

Comparison of the mean values for the different components of *leucaena* (Table 1.9.2.1) with the mean values for the different legume seeds (Table 1.7.1; Ash = 37; CP = 283; EE = 77; Fibre = 74 g kg^{-1}.) shows that the fibre is a factor of

Fig. 1.9.3.1 Biochemical degradation of mimosine



two higher for *leucaena*. The ether extract is considerably lower for *leucaena* than for the legume seeds but the influence of two or three seeds on this value is considerable (Table 1.7.1). The proximate composition of *leucaena* depends very much on the method of harvest and on the care taken in separating stock from leaf. The high ash content in the sample of *leucaena* from Malawi has been attributed to the incorporation of soil during harvesting (D'Mello and Taplin, 1978). The amount of stock material in the leaf will increase the fibre content of the leaf meal, as will cutting at more mature stages of growth (Adeneye, 1979). Variation in the fibre content will have a greater effect on the nutritional characteristics for monogastric animals fed *leucaena* than for ruminants.

The protein content of *leucaena* is higher than that for Lucerne (D'Mello and Taplin, 1978) however the balance of amino acids in the protein of both are similar but the concentrations are lower than that for soya beans. The amino acid content, expressed in terms of the nitrogen content, show that both *leucaena* and lucerne yield 77g amino acids for each 16g of nitrogen (ie. 100g crude protein) while soya beans yield 97g per 16g nitrogen. Obviously there is a higher proportion of non-protein nitrogen present in *leucaena* than in soya beans. One point of note is the high value of isoleucine reported in LLM and *leucaena* seeds (NAS, 1977; D'Mello and Thomas, 1978; Ekpenyong, 1986). The high values reported are likely (in the case of D'Mello and Thomas, certainly) due to the simultaneous elution of the non-protein amino acid mimosine with isoleucine during chromatographic analysis (D'Mello, 1973; Acamovic and D'Mello, 1981).

Leucaena leaf meal is also a good source of pigments and B-carotene (D'Mello and Taplin, 1978), the vitamin A precursor, and therefore has been studied as a potential pigmenting foodstuff particularly for chicks and laying hens (Taplin; D'Mello and Phillips, 1981; Berry and D'Mello, 1981; D'Mello and Acamovic, 1985). From these studies it has been demonstrated that *leucaena* is an effective pigmenting foodstuff, both for egg yolks and carcasses, when included in chick and hen diets at levels from 5 to 250 g kg^{-1}.

1.9.3 Toxic and antinutritional components in *Leucaena*

The toxic component of most interest and intensely studied is the non-protein amino acid mimosine ((S)-B-[N-(3-hydroxy-4-pyridone)]- a-aminopropanoic acid) which under various conditions decomposes to give 3,4-DHP (3-hydroxy-4(1H)-pyridone) (Fig. 1.9.3.1). The abbreviated form, 3,4-DHP, is derived from terminology where the compound was known as 3,4-dihydroxypyridine.

The biosynthesis of mimosine in the plant has been shown to occur from aspartate via lysine, for synthesis of the pyridone ring (Hylin, 1964; Notation and Spencer, 1964; Tiwari and Spencer, 1965) and from serine, via its O-acetyl

derivative, for the alanyl side chain (Tiwari, Penrose and Spencer, 1967; Murakoshi, Kuramoto, Haginiwa and Fowden, 1972). The use of these amino acids in the synthesis of mimosine is not reflected in reduced concentrations of lysine and serine in the protein of LLM as compared to Lucerne but aspartic acid levels are considerably lower in LLM than Lucerne (D'Mello and Taplin, 1978). This may be due to its use for mimosine synthesis.

The structural similarity of mimosine to other amino acids and amines, particularly 3,4-dihydroxy-phenylalanine (DOPA) (Mostad, Romming and Rosenqvist,1973; Mostad, Rosenqvist and Romming, 1974; Fig. 1.8.3.1) may endow it with some of its toxic properties. The S configuration (Beyerman, Maat and Hegarty, 1964) is also highly likely to influence the toxicity of mimosine. These aspects will be reviewed later.

The degradation of mimosine occurs under the action of rumen microorganisms, (Hegarty, Schinckel and Court, 1964; Shiroma and Akashi, 1976; Tangendjaja, Hogan and Wills, 1983) endogenous plant enzymes (Smith and Fowden, 1966; Lowry, Tangendjaja and Tangendjaja, 1983, Tangendjaja, Lowry and Wills, 1984) chemically, (Hegarty, Court and Thorne, 1964; Wills and Tangendjaja, 1981; Acamovic, D'Mello and Fraser, 1982) thermally, (Adams, Cristol, Anderson and Albert, 1945; Matsumoto, Smith and Sherman, 1951; Wood and Carter, 1983) and by ensilage of the fresh material (Lyon, 1985; Lin, Huang and Huang, 1985).

Hydrolysis and enzymic degradation has been reported to give ammonia and pyruvate from the alanyl side chain while rumen degradation has been shown to give the glycoside of 3,4-DHP as well as the free 2,3- substituted isomer (Smith and Fowden, 1966; Hart, Hoffman, Lamberton and Richards, 1977; Tangendjaja and Wills, 1980; Ford, Megarrity and Meehan, 1984). During enzymic degradation in the presence of methanethiol the alanyl side chain is preserved as S-methylcysteine (Murakoshi, Kuramoto, Haginawa and Fowden, 1970). Recent work (Tangendjaja, Lowry, and Wills, 1986) has shown that an enzyme extracted from fresh *leucaena* leaf is a potent degradative agent for mimosine at pH 8 and a temperature of 45°C. The enzyme, mimosinase, has a RAM of 140,000 and is inhibited by the presence of tyrosine.

1.9.3.1 Toxicity of mimosine

There are a number of physiological effects that occur when mimosine is ingested by various animals. The extent of the effect is dependent on the quantity of mimosine ingested related to body size and ability of the animal to detoxify mimosine. If growth rate is the characteristic observed then rabbits are least susceptible followed by chickens, goats, sheep and cattle. Consumption rates of mimosine (g kg^{-1} body weight day^{-1}) at which growth is adversely affected

decreases from 0.21 for rabbits to 0.12 for sheep (Szyszka, ter Meulen and El-Harith, 1983; Szyszka, ter Meulen, Cheva-Isarakul, Posri and Potikanond, 1984). In one experiment with poultry (Springhall, 1965) injection of mimosine at a level of about four times that of sheep produced no adverse effects. A single 1g dose of mimosine given to a six month old cockerel produced no deleterious effects and recovery of only 4.2% of the administered mimosine was obtained. This was excreted solely in the urine and no 3,4-DHP was detected in either urine or faeces (Springhall, 1965). It was concluded that fowl could metabolise mimosine better than rats and ruminants. A more recent experiment with chicks (Librojo and Hathcock, 1974) showed that chicks fed *leucaena* or injected with mimosine, excreted mimosine and 3,4-DHP in the urine. Neither mimosine nor 3,4-DHP were detected in the plasma but two unidentified phenolic compounds were found in the urine and were postulated to be mimosine analogues. It was concluded that mimosine was partly degraded by the chick to 3,4-DHP and that 3,4-DHP was not degraded further. Although mimosine is structurally similar to other aromatic amimo acids it has not been demonstrated that it is incorporated into naturally occuring proteins or peptides although it has been found (ter Muelen, Pucher, Szyszka and El-Harith, 1984) in the tissues of chicks fed leucaena.

The most obvious physiological effect which can be directly attributed to mimosine, when sufficiently high levels are ingested, is depilation. Depilation occurs in animals in which the hair or wool is in the anagen phase when mimosine is ingested (Crounse, Maxwell and Blank, 1962). The loss of hair has been reported for mice (Crounse, Maxwell and Blank, 1962; Panaretto, Tunks and Munro, 1978), rats (Matsumoto, Smith and Sherman, 1951; Castillo, Aglibut, Gerpacio, Gloria, Gatapia and Resurreccion, 1964), horses, cattle, pigs (Mullenax, 1963; Oakes, 1968; Holmes, 1979; Holmes, 1981) and humans (Lowry, Maryanto and Tangendjaja, 1983). Sheep have been shown to lose their wool as a direct result of the ingestion (ca. 300mg kg^{-1} d^{-1}) or intravenous infusion (ca. 80mg kg^{-1} d^{-1}) of mimosine (Hegarty, Schinkel and Court, 1964; Reis, Tunks and Chapman, 1975; Reis Tunks and Hegarty, 1975). (Ingestion of 300mg of mimosine corresponds to ingestion of 10g of *leucaena* dry matter). The effects of the fairly low levels of mimosine on loss of wool instigated various research programs for a number of reasons. The temporary but complete loss of wool from sheep made them extremely susceptible to environmental conditions, therefore work has been carried out to minimize the effects of mimosine on sheep as well as other animals, by reduction in intake. The easiest way of reducing intake of mimosine is to limit the supply of *leucaena*, however this is not readily applicable to ad lib fed forage. An attractive technique, particularly for forage, is to genetically manipulate *leucaena* cultivars to produce low mimosine varieties however this method is not without problems (Hutton, 1985).

Reduction of the mimosine content of *leucaena* is another alternative. This can be achieved by the action of the endogenous enzymes in *leucaena*, on mimosine thus degrading it to 3,4-DHP (Fig. 1.9.3.1; Lowry, Maryanto and Tangendjaja, 1983; Tangendjaja, Lowry and Wills, 1984; 1986) which is less toxic although not non-toxic (see later). The endogenous enzymes are released and degrade mimosine during disruption of the *leucaena* cells.

Goats and steers which were fed *leucaena* coped better with mimosine and 3,4-DHP if their diets were supplemented with oat chaff (steers) and molasses and straw (Elliott, Brandon, Kenny and Evans, 1984; Elliott, Norton, Milton and Ford, 1985). Both mimosine and 3,4-DHP excretion in the urine decreased on supplementation and serum thyroxine (T4) levels in goats correlated inversely with 3,4-DHP in the urine. Adequate supplementation of diets with molasses may assist in reducing the toxicity caused by the ingestion of *leucaena* by ruminants. The mechanism by which this is accomplished is likely to be due to the increased excretion of mimosine and 3,4-DHP which prevents absorption of 3,4-DHP thus preventing interference in thyroid function and a reduction in serum T4 levels (Elliott, Norton, Milton and Ford, 1985).

A considerable amount of work has been conducted on the effects of mimosine ingestion on the composition and quality of wool produced (Gillespie, Frenkel and Reis, 1980). Particular interest has been expressed with regard to the effect of mimosine on the tyrosine content of the wool because of the structural similarity (Fig. 1.9.3.1) of the two amino acids (Frenkel, Gillespie and Reis, 1975; Gillespie, Frenkel and Reis, 1980).

Another area which has aroused considerable interest was the possibility of using mimosine to chemically defleece sheep. To this end mimosine and a wide variety of mimosine analogues were synthesised (Spenser and Notation, 1962; Harris, 1976; Harris and Teitei, 1977; Stewart, 1978; Teitei and Harris, 1979) and used as test substances in an attempt to elucidate the mechanism of toxicity of mimosine and its degradation products (Ward and Harris, 1976; Hegarty, Lee, Christie, De Munk and Court, 1978; Panaretto, Tunks and Munro, 1978; Stunzi, Perrin, Teitei and Harris, 1979). From work on DNA synthesis in-vitro on sheep skin slices it was established that the 3-hydroxy-4-oxo moiety in the pyridine ring was essential for inhibition of DNA synthesis while the attached side chain modified the activity of the test compounds (Ward and Harris, 1976). Other workers (Hegarty, Lee, Christie, De Munk and Court, 1978) concurred on the necessity of the 3-hydroxy-4-oxo grouping to produce toxic effects with mouse bone marrow cells but also found that only 3-hydroxy-4-oxo pyridine molecules with alanyl or 2-aminoethyl substituents on the ring nitrogen were toxic. These workers also

found that the racemic mixture of mimosine was as toxic as the naturally occuring S stereomer.

Other effects attributed to the consumption of mimosine include lethargy, cataract formation, reduction in fertility, goitre, osophageal and leg lesions, paralysis and in extreme cases, death (Matsumoto, Smith and Sherman, 1952; Hylin and Lichton, 1965; Joshi, 1968; Wayman, Iwanaga and Hugh, 1970; Hegarty, Court, Christie and Lee, 1976; Jones, Blunt and Nurnberg, 1978; Reis, Tunks and Chapman, 1975; El-Harith, Schart and ter Muelen, 1979). Goitre, lethargy, hair loss, reduced fertility and paralysis are temporary effects which are remedied by reducing mimosine intake.

1.9.3.2 Biochemical properties of mimosine

Mimosine has been shown to affect the activity of various enzymes in-vitro and in-vivo. The mechanism by which mimosine interferes in enzyme activity is complex and appears to depend on the system in which studies are conducted (Hegarty, Lee, Christie, De Munk and Court, 1978). The two major factors postulated to account for the effects on enzymes are that chelation of metal ion co-factors and/or steric effects, caused by the structural similarity of mimosine to the normal substrates of the appropriate enzymes, occur.

The chelation of metal ions, particularly iron, by mimosine is well documented. This very property has been used for about 35 years for the estimation of mimosine in biological material and as a potential method of reducing the adverse effects of mimosine when ingested by animals (Matsumoto and Sherman, 1952; Matsumoto, Smith and Sherman, 1952).

Iron (III) ions have been shown to form stable chelates with mimosine (Tsai and Ling, 1973). The coordination number of Fe(III) in these chelates is six and the chelates formed are violet in colour. The Fe(III) ion is octahedrally coordinated to mimosine in a bidentate fashion through the ring oxygen atoms giving the violet colour commonly produced with Fe(III) and phenols at low pH. Studies on the stability of mimosine complexes with various metal ions (Tsai and Ling, 1973) show that stability decreases in the order Fe(III), Al(III), Cu(II), Pb(II), Zn(II), Ni(II), Co(II), Ca(II), Mg(II). Mimosine has also been shown to chelate Sn(II) and Cd(II) in a similar dimeric form to Cu(II) (Hashiguchi and Takahashi, 1977; Stunzi, Perrin, Teitei and Harris, 1979). The binding strength of mimosine for metals is greater than that of other amino acids (Stunzi, Perrin, Teitei and Harris, 1979) and therefore sequestration of metals required by enzymes will inhibit the activity of these enzymes. The Fe(II) chelate of mimosine was found to be unstable. This finding was significant from the point of view that Fe(II) was considered to be a suitable supplement for *leucaena* diets to chelate mimosine

thus reducing the toxicity. Oxidation of Fe(II) to Fe(III) which then chelated mimosine seems to be the most likely explanation as to the effectiveness of Fe(II) salts in the reduction in toxicity of *leucaena* based diets. In experiments with young chicks Fe(II) and Al(III) supplementation of diets containing LLM (150g/kg) increased mimosine excretion rates presumably due to chelation effects (D'Mello and Acamovic, 1982).

In experiments with young mice (Hashiguchi and Takahashi, 1977) only Fe(II), injected peritoneally with mimosine, pervented loss of hair. Cu(II) and Zn(II) injected in the same way had no effect although the lack of effect may have been due to the lower metal ion to mimosine ratio.

Mimosine has been shown to competitively inhibit tyrosinase (tyrosine aminotransferase, EC 2.6.1.5) in-vitro at concentrations above 10uM when the analagous amino acid DOPA was used as the substrate. At a concentration of 300μM tyrosine activity was reduced to 10% of its original activity. The inhibitory effects of mimosine were reduced in the presence of Fe(III), Al(III) and Cu(II) ions; Fe(III) being the most effective. The effectiveness of metal ions on reversal or prevention of inhibition of tyrosinase by mimosine was directly related to the stability constants of the chelates. Dopamine B-hydroxylase (dopamine B monooxygenase, EC 1.14.17.1), the enzyme responsible for the oxidation of dopamine to nor-epinephrine, was found to be inhibited uncompetitively by mimosine, mimosineamine and mimosinic acid (Hashiguchi and Takahashi, 1977). The effectiveness of inhibition did not depend on the structural similarity of the test compounds to dopamine or tyramine, the usual substrates for dopamine B-hydroxylase.

Mimosine has been shown to have biocidal properties (Mendoza and Ilag, 1980; Serrano, Ilag and Mendoza, 1983). During studies on the biocidal properties of mimosine (Serrano, Ilag and Mendoza) it was demonstrated that Fe(III) and Al(III) were effective in reducing the antibiotic effects on Sclerotium rolfsii Sacc. whereas Fe(II) and Cu(II) had little effect. Structural analogues of mimosine antagonised its biocidal effects on S. rolfsii. During these studies on the biocidal properties of mimosine on S. rolfsii, enzymes which showed reduced activity in the presence of mimosine included aspartate aminotransferase (aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1), polyphenol oxidase (monophenol monooxygenase, EC 1.14.18.1) and a-amylase, (a-amylase, EC 3.2.1.1). Polyphenol oxidase, also known as tyrosinase (catechol oxidase, EC 1.10.3.19, from melanoma cells and mushrooms has also been shown to be completely inhibited by mimosine (Prabhakaran, Harris and Kirchheimer, 1969a) while that from bananna (Tsai, 1961) was only slightly inhibited in the presence of mimosine. The inhibition of tyrosinase and tyrosine decarboxylase (Crounse,

Maxwell and Blank, 1962) in melanomas has been postulated as being associated with the mechanism by which tyrosine requiring tumours are inhibited by mimosine (Prabhakaran, Harris and Kirchheimer, 1969b; Prabhakaran, Harris and Kirchheimer, 1973; DeWys and Hall, 1973a; DeWys and Hall, 1973b).

The effect of incubation of pyridoxal phosphate with mimosine prior to incubation with tyrosine and bacterial tyrosine decarboxylase (EC 4.1.1.25), a pyridoxal phosphate requiring enzyme, showed that the enzyme was considerably inhibited confirming that pyridoxal phosphate-mimosine complexes are formed (Grove, Ballata, Eastmo and Hwang, 1978). The same workers suggested that tyrosine-mimosine complexes are produced by preincubation of tyrosine and mimosine. Under these conditions decarboxylation was faster, in the presence of tyrosine decarboxylase, than for tyrosine alone. The reduction in pyridoxal phosphate in the presence of mimosine in vivo in the rat has been demonstrated by increased cystathionine in the urine (Grove, Ballata, Eastmo, and Hwang, 1978).

Mimosine has also been shown to inhibit aromatic-L-amino acid decarboxylase (EC 4.1.1.28) and alkaline phosphatase (EC 3.1.3.1) from bovine and mouse kidney respectively (Chang, 1960; Lin, Lin and Ling, 1963). Supplementation of the reaction mixture with Mg(II) completely removed the inhibitory effect of mimosine on alkaline phosphatase (Chang, 1960) while pyridoxal phosphate greatly reduced the effects of mimosine on aromatic-L-amino acid decarboxylase (Lin, Lin and Ling, 1963). The effect of mimosine on enzymes in-vivo in rats showed that the enzyme activities of aromatic-L-amino acid decarboxylase, aspartate amino transferase and glutamate decarboxylase (EC 4.1.1.15) were not significantly reduced (Lin, Lin, Ling and Tung, 1967).

Inhibition of the incorporation of thymidine in-vitro in mouse bone marrow cells, sheep skin slices and H Ep-2 (human epedermoid carcinoma) by mimosine has also been demonstrated (Tsai and Ling, 1971, 1972; Ward and Harris, 1976; Hegarty, Lee, Christie, De Munk and Court, 1979). Mouse bone marrow cells were also very susceptible to mimosinamine, the methyl ester of mimosine, and the racemates of mimosine, 2-methylmimosine and isomimosine (Hegarty, Lee, Christie, De Munk and Court, 1979).

Other workers have shown (Reisner, Bucholtz and Ward, 1979) that DNA, RNA, cell division and protein synthesis in paramecium were all inhibited in the presence of mimosine. These workers suggested that the mechanism by which the inhibition occurred was complex but postulated that mimosine may form ternary complexes with DNA and DNA polymerase thereby interfering with RNA polymerase-DNA complex formation.

The inhibition of aspartate aminotransferase by mimosine in mung beans was not observed although growth of these were depressed considerably (Smith and Fowden, 1966). Growth of beans was restored on supplementation with Fe(II), Fe(III) and pyridoxal phosphate (Smith and Fowden, 1966; Ling, Wen and Ling, 1969). Aspartate aminotransferase from porcine heart, on the other hand, has been shown to be inhibited by mimosine with pyridoxal phosphate reducing inhibition (Lin, Shih and Ling, 1962a). The antagonistic effect of pyridoxal phosphate towards mimosine may be due to complex formation between mimosine and the pyridoxal phosphate moiety of aspartate aminotransferase (Lin, Shih and Ling, 1962b). The discrepancy between the effect of mimosine on fungal and porcine aspartate aminotransferase and that obtained from mung beans may indicate a stronger affiliation between the pyridoxal phosphate and the apoenzyme of mung beans than for the fungal and porcine derived enzyme.

1.9.3.3 The toxicity of 3-hydroxy-4(1H)-pyridone

The presence of 3,4-DHP in animals is due to the enzymic degradation of mimosine by microbes present in the animal and/or endogenous enzymes in the *leucaena* plant. The presence of mimosinase or similar enzymes in *leucaena* seeds has yet to be ascertained. The main effect of 3,4-DHP on cattle, sheep, goats, rats and mice is to produce hyperplastic goitres (Jones, Blunt and Holmes, 1976; Hegarty, Court, Christie and Lee, 1976; Hegarty, Lee, Christie, Court and Haydock, 1979; Christie, Lee and Hegarty, 1979). A notable effect of the goitrogenic properties of 3,4-DHP is the reduction in serum thyroxine (3,5,3',5'-tetraiodothyronine; T4) levels in mice, cattle and goats (Christie, Lee and Hegarty, 1979; Holmes, 1981; Jones and Megarrity, 1983; Jones and Hegarty, 1984). The decrease in serum T4 levels has been shown to be accompanied by a simultaneous increase in serum triiodothyronine (T3) uptake in mice (Christie, Lee and Hegarty, 1979). Dietary supplementation of iodine does not alleviate the reversible reduction of serum T4 (Jones, Blunt and Nurnberg, 1978). Injections of thyroxine alleviates the reduction in serum T4 levels and prevents the formation of hyperplastic goitres but thyroxine injections do not moderate the other adverse effects of 3,4-DHP however (Megarrity and Jones, 1983).

Although 3,4-DHP has been shown to be a potent goitrogen, its conjugated form DHP-3-O-glucuronide (DHPOG) is less potent particularly if given intravenously. If given intragastrically to mice, DHPOG was found to be only slightly less potent as a goitrogen, however blood DHP levels were not monitored during these experiments (Hegarty, Lee, Christie, Court and Haydock, 1979) and it may be that DHPOG was degraded to DHP prior to absorption into the blood. Thus 3,4-DHP would, in fact, have been the goitrogen. 3,4-DHP shows similar characteristics to resorscinol (1,3-dihydroxybenzene) and methyl thiouracil in

their ability to cause goitre by inhibiting iodine binding rather than the iodine trapping step in the thyroid (Hegarty, Lee, Christie, Court and Haydock, 1979). Other work has shown that 3,4-DHP, but not its O-glucose nor its O-sulphate conjugates, is an inhibitor of peroxidases (Christie, Lee and Hegarty, 1979). It seems reasonable that 3,4-DHP will therefore inhibit, in-vivo, thyroid peroxidase which is an essential enzyme in the biosynthesis of iodothyronines (Christie, Lee and Hegarty, 1979). The 3-hydroxy group is necessary for potent antithyroid activity but other work using the pyrone analogue (Lee, Hegarty and Christie, 1979; Bhathal, Lo, Hegarty and Harris, 1984) has indicated that the ring nitrogen is also important since 3-hydroxy-4-pyrone is only weakly goitrogenic although it is a strong inhibitor of thyroid and other peroxidases (Lee, Hegarty and Christie, 1979).

Work has shown that 3,4-DHP is also a more potent irreversible inhibitor of catechol methyltransferase (EC 2.1.1.6; COMT) than 3-hydroxy-4-pyrone (Borchardt, 1973). The enzyme COMT is involved in the deactivation of catecholamines (Fig. 4.4.4) thus the presence of 3,4-DHP may tend to increase activity of the animals and also affect food intake due to abnormal concentrations of the biogenic amines within animals consuming mimosine and/or 3,4-DHP. A recent proposal (Megarrity and Jones, 1983) has suggested the chelation of zinc by 3,4-DHP may be partly attributable to the deleterious effects caused by the consumption of mimosine or 3,4-DHP.

1.9.3.4 Other toxic/antinutritional compounds in *leucaena*.

Leucaena leaf and seeds contain galactomannan gums, rhamnose, arabinose, xylose and glucose (Bailey, 1971; Dea and Morrison, 1975; Dey, 1978; Lesniak and Liu, 1981a; Lyon and Kohler, 1981). There is no indication in the literature regarding the similarity or dissimilarity of the galactomannans isolated from the seeds and the leaf. Galactomannan from the seeds has been reported to have a fairly regular distribution of a-D-galactosyl residues and has been found to be more susceptible to degradation by B-D-mannanase than other galactomannans with the same mannose to galactose ratio (Dey, 1980). The seed galactomannan is also readily hydrolysed during germination by an enzyme called galactomannan depolymerase (Hylin and Sawai, 1964). Although this enzyme does not catalyse the production of reducing sugars (Hylin and Sawai, 1964) it is likely to reduce the haemolytic effects of the endogenous galactomannans (Lesniak and Liu, 1981b). The haemolytic effects of *leucaena* galactomannans have been shown to be more prominent as the mannose : galactose ratio increases from about 1.33:1 to 3.03:1 on treatment with a-galactosidase. Galactomannans from different sources showed a similar increase in haemolytic behaviour in direct relationship to their mannose : galactose ratios (Lesniak and Liu, 1981b).

Other toxic components in *leucaena* include polyphenols and their derivatives (Ranganathan and Nagarajan, 1980; Fraser and D'Mello, 1981; D'Mello and Fraser, 1981; Lowry, Cook and Wilson, 1984; Telek and Garcia, 1984). Seven glycosides of quercitin and myricitin have been identified in *leucaena* leaf at a combined concentration of 35-60g kg^{-1} dry matter (Lowry Cook and Wilson, 1984). The use of HPLC to detect these flavonoids has been proposed in order to assess the quality and cultivar of *leucaena* as well as to detect any contamination of grass meal with LLM (Lowry, Cook and Wilson , 1984). The total tannin content, which is likely to include flavonols, varies between about 20 and 34g kg^{-1} depending on the quality of the leaf and on the method of analysis (D'Mello and Fraser, 1981; Fraser and D'Mello, 1981). Some of the phenolic components have been shown, by HPLC, (Kufidis, personal communication) to include proanthocyanidins such as catechin, epicatechin, cyanidin and procyanidin B-2. These phenolic compounds, in common with mimosine and 3,4-DHP, will chelate metal ions such as Fe(III).

1.10 JACK BEANS [*Canavalia ensiformis* (L.) DC.]

Jack beans (JB) are New World legumes of the Fabaceae family. There are some 50 species of *Canavalia* of which JB is one. JB originated in South America but is currently grown in Central America and other parts of the tropics (Duke, 1981; Smartt, 1985; C. Kessler, personal communication).

Growth of JB occurs in adverse soil and climatic conditions where temperatures range from 15-32°C, rainfall from 900-1200 mm $annum^{-1}$ and soil pH between 4.6 to >7.5 although best growth is reported for pHs between 5 and 6 (NAS, 1979; Duke, 1981; Smartt, 1985; Kessler, personal communication). JB plants are more capable of withstanding waterlogging and drought conditions and increased salinity than other legume plants (Duke, 1981).

Yields of green matter which is considered to be a suitable forage especially when dry, and seed can be as high as 60 and 17 tonnes ha^{-1} respectively when conditions are good (Duke, 1981; Dixon, Escobar, Montilla, Viera, Carabano, Mora, Risso, Parra and Preston, 1983). Seed yields of up to 1.9 tonnes ha^{-1} have been obtained in the Yucatan area of Mexico where soil pH is high and the ground is extremely rocky (Kessler, personal communication). Seed weight is much greater than that for lupins and *leucaena* at about 1700g per thousand seeds (Kessler, personal communication).

1.10.1 Uses of Jack Beans

JB are cultivated primarily as a green manure, cover crop, forage and vegetable (Duke, 1981). Use as a vegetable is limited due to toxic components which are

present in the bean. The adverse effects can be reduced by washing, prolonged cooking in water and salt solution and by fermentation (Duke, 1981; Smartt, 1985). Another use which has not been referred to in the literature is the provision of materials such as lectins, canavanine, urease and various other enzymes for chemical, biochemical and medical uses.

The seeds may have potential as a source of leguminous starch with which they are relatively well endowed (Rosenthal, 1970a; Bailey, 1971; Molina and Bressani, 1975). Another potential use of JB is as a supplement to animal diets while the forage has high potential as a source of protein concentrate for animals (Molina, Argueta and Bressani, 1974; Molina and Bressani, 1975; Jokl and Carlsson, 1984).

1.10.2 Composition of Jack Beans

There is a very limited amount of data available on the proximate analysis of JB presumably because of the problems associated with the consumption of these seeds.

The proximate composition of JB compares very favourably with that for other legume seeds (Table 1.7.1) and LLM (Table 1.9.2.1). The lipid value is higher and the fibre content, lower for JB than for LLM. The gross energy is, however, lower for JB than for LLM or the other legume seeds (Table 1.7.1). The protein content (calculated as Nx6.25) is likely to be substantially higher than the actual value due to the presence, as with lupins and LLM, of non-protein amino nitrogen. Therefore the use of 6.25 as the conversion factor does not give the correct protein value. The starch and amylose contents are reported to be about the same (295 g/kg) as those for other beans (Molina and Bressani, 1975; Reddy, Pierson, Sathe and Salunkhe, 1984; Rosenthal, 1970a). JB starch is much more homogeneous than cereal starches and is considerably more resistant to degradation by a-amylase (Rosenthal, 1970a). The indispensable amino acid content and profile of JB is good except for the deficiency of sulphur amino acids, the content of which, is about half that required by poultry (Table 1.7.3; Vaidehi and Shivaleela, 1984; D'Mello, Acamovic and Walker, 1985). Notably the arginine concentration (15.6g kg^{-1}; D'Mello, Acamovic and Walker, 1985) is about half that for other legume seeds (Table 1.7.3) which have approximately the same protein content. This may be due to the presence of canavanine which interferes in the synthesis of arginine. This assumption is supported by the recent observation that clover seeds with high canavanine contents have a lower arginine concentration than seeds with low canavanine contents (Natelson and Bratton, 1985a).

Table 1.10.1 The proximate analysis of Jack Beans (dry matter basis) from various sources and analysed in different laboratories.

crude protein	lipid	fibre	ash	NFE*	gross energy	ref.
(g kg^{-1})					(MJ kg^{-1})	
375.5	31.0	24.0	15.0	554.5	nd	1
310.0	40.0	nd	nd	nd	11.7	2
274.4	29.1	82.9	35.8	577.8	16.3	3
295.0	19.0	90.0	25.0	571.0	18.7	4
308.1	21.2	92.4	28.0	470.3	19.0	5
347.7	34.9	109.3	30.2	477.9	nd	6

Ref. 1: Vaidehi & Shivaleela, 1984; 2: Dixon, Escobar, Montilla, Viera, Carabano, Mora, Risso, Parra & Preston, 1983; 3: Ellis & Belmar, 1985; 4: Duke, 1981; 5: D'Mello, Acamovic & Walker, 1985; 6: Molina & Bressani, 1975.

1.10.3 The value of Jack Beans as a feedingstuff.

The seeds from JB are a rich source of protein and non fibrous carbohydrate (Table 1.10.1) and therefore are a potentially useful ingredient in monogastric and ruminant diets. Inclusion of raw JB at 500g kg^{-1} in diets for conventional quail induced 100% mortality while inclusion levels of up to 300g kg^{-1} for chicks produced extremely poor growth rates and efficiency of food conversion, gastric lesions and 50% mortality (Jayne-Williams, 1973; 1978; Dixon, Escobar, Montilla, Viera, Carabano, Mora, Risso, Parra and Preston, 1983; Belmar, Ellis and Laviada, 1985). Similarly, rats fed diets containing up to 300g raw JB kg^{-1} are reported to have shown no toxic effects while diets containing more than 500g kg^{-1} induced gastric lesions and killed all rats (Orru and Demel, 1940). Inclusion of JB at 460 g kg^{-1}, in other work with rats, produced very poor growth rates but no deaths were reported (Borchers and Ackerson, 1950). Cooking JB in all cases alleviated the mortality but only partially reduced the growth inhibition caused by inclusion of JB and which can be attributed to con A (Jayne-Williams, 1973; 1978). Chicks fed diets containing 140 and 280 g of autoclaved JB kg^{-1} diet had lower growth rates, food intake and efficiency of food utilisation and nitrogen retention than birds fed a soya bean control diet (D'Mello, Acamovic and Walker, 1985). Similar but less severe growth depressions, relative to controls, have been observed in experiments by other workers where cooking method and time of cooking were varied (Belmar, Ellis and Laviada, 1985).

The AME and AMEn (AMEn : nitrogen corrected AME) of JB for chicks has been determined to be 11.8 and 12.0 MJ kg^{-1} respectively (D'Mello, Acamovic and Walker, 1985). This is comparable with the AME for winged bean, higher than that reported for lupins but lower than for maize (12.1, 11.0 & 14.4 MJ kg^{-1} DM) respectively; (D'Mello, Acamovic and Walker, 1983 and see section 1.11.3). The PER of raw and cooked JB is low (Molina and Bressani, 1975). The PER of cooked JB was less than half that for casein while the PER of raw JB was essentially 0. The PER of JB/semolina diets for rats was low (Vaidehi and Shivaleela, 1984) compared with a diet composed of skimmed milk powder. These workers indicated that the protein quality of JB was poor. They did not supplement the diets with sulphur amino acids, which they acknowledged was low. Amino acid supplementation has been shown to increase the PER of lupins (Schoeneberger, Gross, Cramer and Elmfada, 1982; Table 1.11.3.1). Another major factor which must be taken into account is the presence of non protein nitrogen in JB. The nitrogen content of the JB used in the determination of PER was 56 g kg^{-1}. If the canavanine content is assumed to be 40 g kg^{-1} then the nitrogen contribution from canavanine is 14 g kg^{-1}. Assuming that other non protein nitrogen sources are negligible then the protein nitrogen content is 42 g

kg^{-1} (ie. about 22% lower than estimated) which will considerably affect the PER of JB.

Laying hens are reported to be less susceptible than broilers to the inclusion of JB in their diets although inclusion levels were low, at 100 and 200 g kg^{-1} of raw and cooked JB respectively (Herrera, Gutierrez, Cupul, Ferreiro, Carabano and Montilla, 1981). Other treatment of JB such as ensilage with ammonia or urea is partially successful in decreasing the toxicity of JB for chicks although autoclaving of the ensiled material was necessary for best results (Montilla, Ferreiro, Cupul, Guttierrez and Preston, 1981; Dixon, Escobar, Montilla, Viera, Carabano, Morra, Risso, Parra and Preston, 1983).

Pigs are highly susceptible to the inclusion of autoclaved JB into their diets. Even at levels as low as 50g kg^{-1}, growth rate is reduced and if the beans are included raw pigs will reject the diets (Dixon, Escobar, Montilla, Viera, Carabano, Morra, Risso, Parra and Preston, 1983; Godoy, personal communication).

A similar animal to the pig (white collared peccary) is reported to be unaffected by inclusion of raw JB at levels up to 150 g kg^{-1} (Dixon, Escobar, Montilla, Vierra, Carabano, Morra, Risso, Parra and Preston, 1983). It is thought that the foregut fermentation in the peccary reduces the toxicity caused by canavanine.

Ruminants are reported to be least affected by the ingestion of JB in which ingested protein has a degradability of about 0.83 and growth tends to be slightly depressed (Dixon, Escobar, Montilla, Vierra, Carabano, Morra, Risso, Parra and Preston, 1983; Pacheco and Rivera, 1985). The lack of effect of JB on ruminants may be due to the fermentation of toxic factors in the rumen.

1.10.4 Toxic components of Jack Beans.

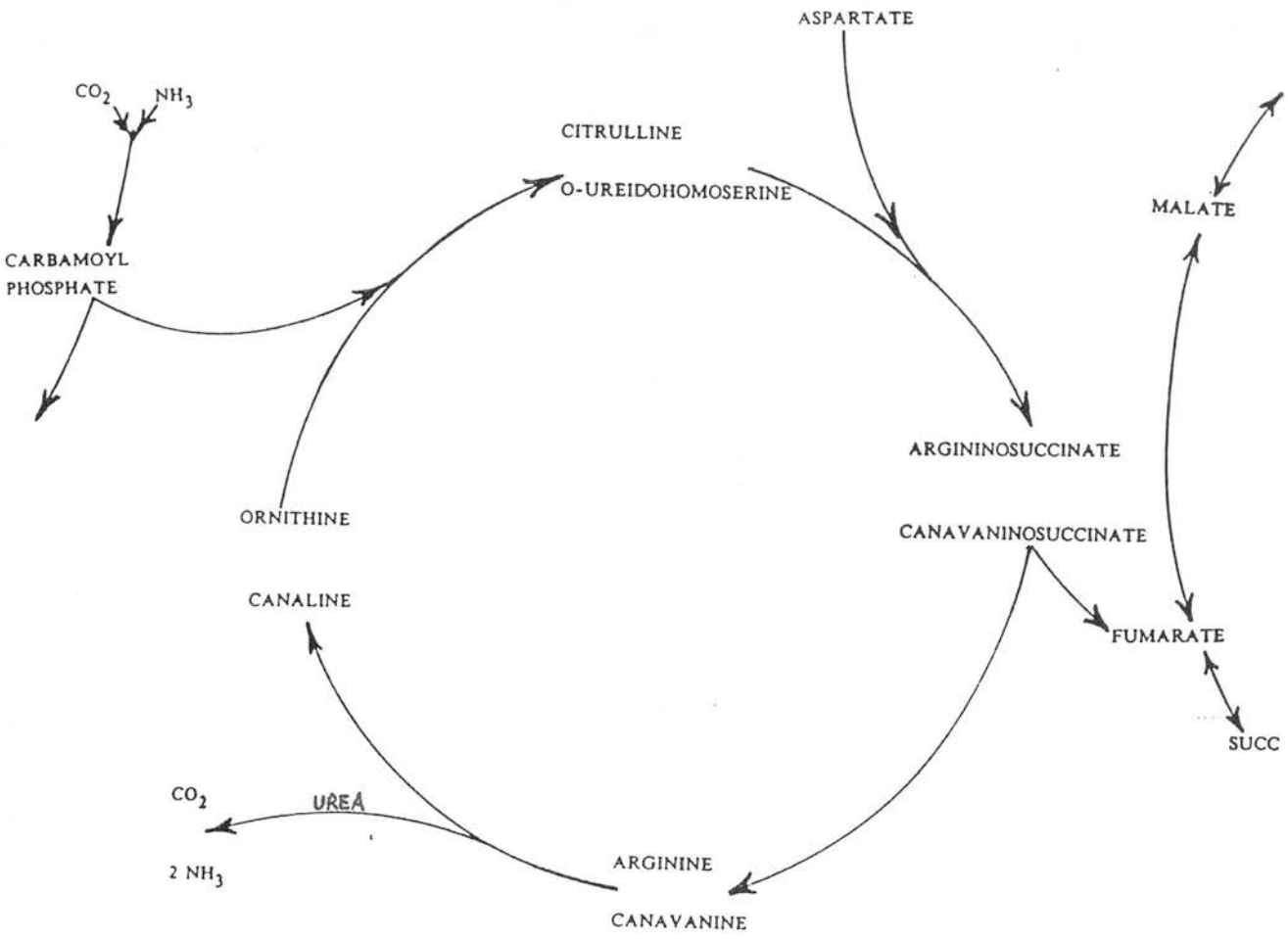
The two major toxic components of JB are considered to be the lectins and canavanine.

Con A is synthesised and accumulates in the protein bodies of the storage-parenchyma cells, in the cotyledons of JB seeds (Herman and Shannon, 1984). It is the major, but not the sole toxic lectin in JB (Carlini and Guimaraes, 1981; Raychaudhuri and Singh, 1986), which has been shown to be formed from the loss of a mannose polysaccharide from the Con A glycoprotien precursor, thus yielding the protein Con A (Herman, Shannon and Chrispeels, 1985). As has been mentioned previously Con A is destroyed by heating thus reducing the toxic effects caused by the consumption of JB (Jayne-Williams, 1973; 1978).

Canavanine is a thermally stable arginine analogue (Fig. 1.8.3.1) found free in JB (Kitagawa and Tomiyama, 1929), has been chemically synthesised from canaline (Nyberg and Christensen, 1957; Rosenthal, Downum and Mattler, 1983) and which has a chemistry similar, although with significant differences, to that of arginine (Greenstein and Winitz, 1961; Boyar and Marsh, 1982). It accounts for more than 95% of the free amino nitrogen and is one of the major nitrogen storage compounds in JB seeds (Johnson, 1956; Rosenthal, 1977; 1977b; Rosenthal and Rhodes, 1984). The biosynthesis, in JB, and degradation, in JB and animals, of canavanine parallels that of arginine (Walker, 1957; Rosenthal, 1982, Milner, 1985) and is summarised in Fig. 1.10.4. The interrelationship between the arginine-urea cycle and the tricarboxylic acid cycle infers that the synthesis of tricarboxylic acid intermediates (Milner, 1985) is likely to be strongly influenced by the presence of canavanine and its various metabolites (Katunuma, Okada, Matsuzawa and Otsuka, 1965). Canavanine and its homologues are also likely to have a similar effect on growth, food intake and tissue amino acid concentrations as have other unconventional amino acids fed to rats (Tews and Harper, 1985a; 1985b).

$^{14}CO_2$ has been shown to be incorporated into canavanine in biological systems via O-ureidohomoserine (Warren and Hunt, 1971; Rosenthal, 1982). Canaline has been shown to be a substrate for the formation of canavanine via O-uriedohomoserine although canaline, produced from the degradation of canavanine, has not been shown to be resynthesised into canavanine. It has been suggested that the synthesis of canavanine is discrete from its degradation (Rosenthal, 1982) as has been thoroughly discussed for other metabolic systems (Oaks and Bidwell, 1970). Both degradation and synthesis of canavanine occurs in the cotyledon of the seed (Warren and Hunt, 1972; Rosenthal, 1982; Downum, Rosenthal and Cohen, 1983; Rosenthal and Rhodes, 1984) and in the leaf (Rosenthal, 1972). Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) is the enzyme which catalyses the degradation of canavanine to urea and canaline (Damodaran and Narayanan, 1940; Downum, Rosenthal and Cohen, 1984). Urease (EC 3.5.1.5) which has pronounced activity in JB and other canavanine containing legumes as well as other members of the Leguminosae (Bailey and Boulter, 1971; Ho and Shen, 1966; Rosenthal, 1974; 1977), then degrades the urea to CO_2 and NH_3, the NH_3 then being utilised for amino acid and protein synthesis (Rosenthal, 1970). The degradation of canavanine in seeds is most prominent as the seeds germinate with no canavanine being found in germinating lucerne seeds (Bell, 1960; Ho and Shen, 1966; Rosenthal, 1970; Rosenthal, 1977). Both urease and arginase activity in JB has been shown to be highest between the 4th and 5th and the 6th and 7th days respectively, of germination (Ho and Shen, 1966).

Fig. 1.10.4 **Diagram of the interaction of canavanine in the arginine urea cycle.**



From: Rosenthal, 1982 & Milner, 1985.

Degradation of canavanine has been shown to give other products such as homoserine, hydroxyguanidine, deaminocanavanine and guaninidine when canavanine was incubated with micro-organisms and arginase (Kalyankar, Ikawa, Snell, 1958; Warren and Hunt, 1971)

The biological effects of canavanine include a reduction of protein and glycoprotein synthesis, inhibition of alkaline phosphatase activity and inhibition of RNA synthesis (Rosenthal, 1977). Canavanine is considered to be involved in the induction or reactivation of a blood disorder, systemic erythematosis syndrome in humans and monkeys (Malinow, Bardana, Pirofsky, Craig and McGlaughlin, 1982; Roberts and Hayashi, 1983).

The toxicity of canavanine is ascribed to its structural similarity to arginine, which is necessary for the synthesis of animal and insect tissue proteins (Hegdekar, 1970; Dahlman and Rosenthal, 1976; Milner, 1985; Visek, 1986). The functions and necessity of arginine for animal growth is the subject of two recent reviews (Milner, 1985; Visek, 1986). Canavanine can readily substitute for arginine in many of its biochemical processes (Walker, 1955; Kalyankar, Ikawa and Snell, 1958; Attias, Schlesinger and Schlesinger, 1969). Canavanine, unlike mimosine, has been shown to be incorporated into proteins at the expense of arginine (Allende and Allende, 1964; Neurath, Wiener, Rubin and Hartzell, 1970; Dahlman and Rosenthal, 1976; Crine and Lemieux, 1982) . A high proportion of the arginine molecules (65-95%) in alkaline phosphate have been shown to be replaced by canavanine. The resultant proteins have been shown to have much reduced activity and also to modify the cleavage of the substrate proteins yielding products which are different from those from their analogous arginine containing substrates (Crine and Lemieux, 1982).

Canavanine containing proteins are thought to be responsible, in some manner, for the occurrence of systemic erythematosis syndrome in monkeys and humans that have consumed lucerne seeds (Malinow, Bardana, Pirofsky, Craig and McGlauchlin, 1982; Roberts and Hayashi, 1983). The incorporation of canavanine into such proteins may cause an alteration of their tertiary structure thus limiting their effectiveness.

The inhibition of various enzymes including alcohol dehydrogenase, B-glucosidase and oxynitrilase (EC 1.1.1.1; EC 3.2.1.21; EC 3.5.5.1 respectively), by canavanine is well known (Tschiersch, 1966; Rosenthal, 1977). N-acetyl-B-glucosaminidase (EC 3.2.1.30) from rats has also been shown to be inhibited (Powell and Riedenberg, 1983). Liver arginase activity is shown to be inhibited in the presence of canavanine although canavanine is degraded by the enzyme to canaline (Fig. 1.10.4; Damodaran and Narayanan, 1940). The significance of this inhibition in animals could be considerable considering the importance of both

arginine and arginase in maintaining normal growth (Rogers and Visek, 1985; Milner, 1985; Visek, 1986).

It is considered that the analogous structure of canavanine with arginine may cause inhibition of arginase by competition between them at the active sites on arginase. This is contrary to the antagonistic effect of lysine on arginine in birds and mammals (Jones, Petersburg and Burnett, 1967; Crine and Lemieux, 1982; Visek, 1986).

Canaline (Fig. 1.8.3.1) is the major part of the canavanine molecule left after treatment with arginase (Fig. 1.10.4) and which is also reported to occur in JB (Rosenthal, 1972). In contrast to arginine, canavanine is relatively stable in alkaline solution but can be degraded using barium sulphate, to canaline (Rinderknecht, 1960; Rosenthal, 1972a). The complete chemical synthesis of the racemate of canaline has also been achieved (Knobler and Frankel, 1958).

The structural similarity of canaline and ornithine has allowed the substitution of canaline instead of ornithine during studies on mammalian enzymes (Walker, 1957; Kekomaki, Rahiala and Raiha, 1969; Rahiala, Kekomaki, Janne Raiana, and Raiha, 1971). From these studies it was shown that the enzymes which were inhibited by canaline were pyridoxal phosphate requiring enzymes such as the transaminases (Katunuma, Matsuda and Tomino, 1964; Rahiala, 1973). Some other reactions catalysed by pyridoxal phosphate were also shown to be inhibited in the presence of canaline (Rahiala, Kekomaki, Janne, Raiana and Raiha, 1971). These include reactions involving the enzymes ornithine decarboxylase (EC4.1.1.17), 5-hydroxytryptophan decarboxylase (EC4.1.1.28) and plasma diamino oxidase (EC 4.1.3.6).

Canaline may exert its toxicity in animals, if it is toxic, by inhibition of the enzymes mentioned above, competition with ornithine in the arginine urea cycle and/or by complexation with pyridoxal phosphate cofactor in a similar manner to mimosine. The canaline pyridoxal phosphate complex is stable between pH 6 and 8.5 as demonstrated by the inability of supplemental pyridoxal phosphate to reactivate rat liver ornithine keto acid aminotransferase (EC 2.6.1.13) in-vitro, and by examination of the spectra of the mixtures of canaline and pyridoxal phosphate (Rahiala, Kekomaki, Janne, Raina and Raiha, 1971; Rahiala, 1973). Canaline is considered (Rahiala, 1973) to form an oxime via the aldehyde group on pyridoxal phosphate thus accounting for the uncompetitive inhibition observed for a-ketoglutarate conversion, and the competitive inhibition of removal of ornithine (Rahiala, 1973).

Other reported (Mears and Mabry, 1971; Duke, 1981) antinutritional and (potentially) toxic components of minor importance in JB include protease

inhibitors, saponins, HCN and the two alkaloids trigonelline (3-carboxy-1-methyl pyridinium hydroxide inner salt) and stachydrine (2-carboxy-1,1-dimethyl pyrrolidinium hydroxide inner salt). The physical and chemical characteristics, synthesis and source of these have been summarised (James, 1950; Marion, 1950; 1950a; Merck Index, 1983). Both the alkaloids are not reported to be highly toxic (Merck Index, 1983) although stachydrine may antagonise tyrosine metabolism due to their analogous structures.

1.11 LUPINS.

Lupinus consists of about 212 species of which 12 are reported to be Old World. *Lupinus* is the only genus of the predominately tropical Fabaceae which has been exploited as a temperate annual grain legume (Pate, Williams and Farrington, 1985). They have been exploited in warmer climates for greater than 4000 years as a nutritious high protein foodstuff but greatest interest, study and use has occurred since the early twentieth century (Gladstones, 1970) when very small numbers of 'sweet varieties' were discovered amongst extremely large numbers of other lupin plants (Gladstones and Francis, 1965). Only three of the species of Mediterranean/African origin have attained full crop status (Gladstones, 1984) and only four of the 1700 named varieties are considered as having most potential for seed production in the United Kingdom and European Economic Community (EEC) countries (Belteky and Kovacs, 1984). The highest rate of production of lupins occurs in the USSR followed by that in Australia with about 80kha planted (Lees, 1984). This is also reflected by the amount of literature published on lupins where the USSR has the highest publication rate and Australia with the next highest. The UK has produced the third largest amount of literature on lupins although this does not reflect on either the amount of work on lupins nor the production rates (Wells, 1984). Lupins are still of minor importance in terms of quantity produced relative to other seeds (Table 1.6.1) and was about 0.5M tonnes in 1982 (Pate, Williams and Farrington, 1985).

There has been increased interest in the production of lupins in Europe in recent years for two prime reasons. Europe, and the UK particularly, depends heavily on imports of soyabeans for protein and vegetable oil. The world-wide shortages in 1973 and consequent increase in prices encouraged EEC countries to be more self sufficient in both these commodities thus alternative protein and oil sources were sought (Belteky and Kovacs, 1984). Initially peas and beans were financially supported by the EEC but recently similar subsidies have been provided for lupins (Belteky and Kovacs, 1984) thus providing impetus for further work and investment.

Details of the botany, genetics, taxonomy and conditions of growth of lupins have been thoroughly described and reviewed (Gladstones, 1970; Gladstones,

1971; Aguilera and Trier, 1978; Belteky and Kovacs, 1984; Gladstones, 1984; Ravelo, 1984; Plitmann and Heyn, 1984; Dunn, 1984).

1.11.1 Uses of lupins

The uses and potential uses of lupins are varied and include ornamental and decorative functions, coffee substitutes, soil improvement, manuring, oil production and by far the most important, as ruminant forage and high protein feedstuff (Gladstones, 1970b; Hill and Arnold, 1975; Hill, 1977; Aguilera and Trier, 1978; Schoeneberger, Gross, Cramer and Elmfada, 1982; Belteky and Kovacs, 1984). The use of whole lupin plant as well as the stubble remaining after harvesting as forage for ruminants is limited due to the development serious nutritional disturbances caused by a disease called lupinosis.

Lupinosis is particularly prevalent in sheep and can cause death (Gladstones, 1970). The disease produces fatty livers and causes liver damage (Allen, Wood, Croker and Hamblin, 1979; Croker, Allen, Petterson, Masters and Frayne, 1979). Lupinosis syndrome is due to a mycotoxicosis caused by two metabolites, phomopsin A and phomopsin B found in the fungus Phomopsis leptostromiformis which grows on stubble and stalks of lupins in moist atmospheres (Culvenor, Beck, Clarke, Cockrum, Edgar, Frahn, Jago, Lanigan, Payne, Peterson, Petterson, Smith and White, 1977; Allen, Wood, Croker and Hamblin, 1979). Injection of the toxin into rats and mice produces lupinosis. *Lupinus albus* (cv. Ultra) has been shown to be less susceptible to fungal infection by Phomopsis leptostromiformis than other species of lupin (Wood and Allen, 1980) and consequently less toxic to sheep. Plant breeding techniques have produced other phomopsis resistant lupins (Allen and Cowling, 1986).

The fungus has been reported not to grow on the seeds of lupins (Gladstones, 1970) and there was little concern about the effects of feeding lupin seed until sheep which were fed fungal affected seed developed the disease (Allen, Moir and Mackintosh, 1983). Recent work has shown that lupin seeds are susceptible to fungal infection, which is highly correlated with rainfall, and that 42 to 61 samples in every 100 tested showed fungal infection with phomopsin contents which varied between <6 to 360ug kg^{-1} of seed (Wood and Petterson, 1985; Petterson, Peterson, Smith, Wood and Culvenor, 1985; Wood and Petterson, 1986). Discoloured seeds have been shown to contain the highest amount of phomopsin A and also to be effected to the greatest extent by the fungus (Wood and Petterson, 1986). Recent reports also indicate that residual seed strongly influences the development of lupinosis in sheep grazed on lupin stubble (Allen and Cowling, 1986).

Fungal growth may limit the production of lupins in Scotland.

Table 1.11.1 Proximate composition of four lupin species. (g kg^{-1} dry matter)

Species	crude protein (N*6.25)	ether extract	Fibre	Ash
L. albus	343-449	82-145	33-195	29-47
L. angustifolius	280-379	53-84	130-273	24-39
L. luteus	360-476	40-71	146-176	40-52
L. mutabilis	317-459	131-231	74-182	30-45

compiled from data from : Hill, 1977; Pearson and Carr, 1976; Hove, King and Hill, 1978; King, 1981; Halvorson, Shehata and Waibel, 1983; Aguilera, Molina and Prieto, 1985; Hale and Miller, 1985; Aguilera, Prieto, Fonolla and Gil, 1986.

1.11.2 Composition of lupin seed

The major attraction of lupins as a crop is the high protein seeds which it produces. The proximate analysis of the most promising species of lupin for the UK are presented in Table 1.11.1. and compares favourably with the data for other legumes (Table 1.7.1) and that presented for LLM (Table 1.9.2.1).

Recent studies on the characterisation of proteins in L. *luteus*, L. *angustifolius* and L. *albus* (Elleman, 1977; Restani, Duranti, Cerletti and Simonetti, 1981; Duranti, Restani, Poniatowska and Cerletti, 1981; Varasundharosoth and Barnes, 1985) has shown that most of the lupin protein exists as albumins and globulins with minor amounts of prolamins and glutelins. L. *albus* and L. *angustifolius* proteins had higher proportions of globulins than albumins whereas L. *Luteus* proteins consisted of a higher proportion of albumins. Of the species and cultivars of lupin studied the protein from L. *angustifolius* (cv. Fest) had the best balance of indispensible amino acids. Of the protein fractions studied, the glutelin fraction of L. *albus* had the best balance and was considered to be an excellent source of indispensible amino acids (Varasundharosoth and Barnes). Sulphur amino acids are the first limiting amino acids in lupins with tryptophan second limiting (Karunajeewa and Bartlett, 1985) and lysine reported to be marginal in some cases, particularly in cultivars with a higher alkaloid component (Gladstones, 1970; Hill, 1977; Barnett and Batterham, 1981; Aguilera, Molina and Prieto, 1985). Although the lysine content of lupins is marginal its availability is reported to be considerably lower than that for soya bean (King, 1981).

The fibre content of lupin (Table 1.11.1) is higher than that for other legume seeds (Table 1.7.1) but in the same range as *leucaena* (Table 1.9.2.1). The majority of the fibre in lupins is found in the testa which comprises between 12 and 32 percent of the seed weight depending on the species and cultivar (Brillouet and Riochet, 1983). The largest carbohydrate component in lupin testa has been shown to be cellulose with hemicellulose providing the majority of the remaining carbohydrate (Bailey, Mills and Hove, 1974; Carre and Brillouet, 1986). The testa accounts for most of the cellulose found in lupins, very little being associated with the cotyledon (Brillouet and Riochet, 1983). The amount of lignin in the seeds is low (ca. 10g kg^{-1} seed) and is partitioned mainly in the testa (Bailey, Mills and Hove, 1974; Brillouet and Riochet, 1983; Reddy, Pierson, Sathe and Salunkhe, 1984; Carre, Brillouet and Thibault, 1985; Carre and Brillouet, 1986). The low lignin content is thus likely to enhance the digestibility of lupins (Minson, 1982).

The lipid is composed primarily (ca. 0.85) of unsaturated fatty acids most of which is (C18:1) oleic acid. The fatty acid content of lupins is similar to that of soya bean but higher in oleic acid and lower in (C18:2) linoleic acid (Hudson,

Fleetwood and Zand-Moghaddam, 1976; Schoeneberger, Gross, Cremer and Elmfada, 1982; Green and Oram, 1983; Eskin and Warenko, 1985). The proportion of total saturated to total unsaturated fatty acids is similar to that for other legume seeds (Salunkhe, Sathe and Reddy, 1983).

Lupins tend to have a high manganese content, the highest reported levels being about 7g kg^{-1}, (Hill, 1977; Oram, David, Green and Read, 1979; Aguilera, Molina and Prieto, 1985; Karunajeewa and Bartlett, 1985) which may limit the incorporation of lupins into pig and poultry diets. Incorporation of L. *albus* (330g kg^{-1}) with a manganese content of 2.3 g kg^{-1} DM into pig diets increased the liver manganese content by 30% above these fed the control diet (King, 1981). The growth rate of pigs fed these lupin supplemented diets were lower than controls but were the same as manganese supplemented diets. The higher manganese contents in L. *albus* does not, therefore, appear to be the cause of lower growth.

Another potentially useful facet of lupins is their content of carotenoid pigments, most of which is zeaxanthin (Hudson, 1979; El-Difrawi and Hudson, 1979). The pigments are likely to colour egg yolks if laying hens are fed lupin seed.

1.11.3 The value of lupin seed as a feedingstuff.

The major interest in lupin seeds is as a supplement to the diets of monogastric animals insead of soya meal and maize. Most work on the nutritive value of lupins has been conducted using poultry, pigs and rats. Most early work on the nutritional quality of lupin seeds is reported to have been carried out in Germany in the 1930s when 'sweeter varieties' (lower alkaloid varieties) were developed (Gladstones, 1970a; Gladstones, 1971; Hill, 1977). Since that time most work throughout the world has been on newer and lower alkaloid varieties.

It is well documented that growth, food intake and efficiency of food conversion (EFC) of monogastric animals is adversely affected by the inclusion of lupin seed in their diets. Generally the species of lupin incorporated into the diet has a variable but small effect if the varieties used are low alkaloid varieties and inclusion levels are below about 200 and 300g kg^{-1} for pig and poultry diets respectively (Erickson and Elliott, 1984a; 1984b; Gladstones, 1984; Karunajeewa and Bartlett, 1985). The AME of lupins for poultry has been determined to be about 11 MJ kg^{-1} dry seed (Guallaume, Chenieux and Rideau, 1979; Halvorson, Shehata and Waibel, 1983) while the digestible energy (DE) for pigs is about 17.6 MJ kg^{-1} (16.5-18.2) which is about the same as soyabean meal (Taverner, 1975; Batterham, 1979; King, 1981; Aguilera, Molina and Prieto, 1985). The AME for pigs is about 16.9 MJ kg^{-1} (Aguilera, Molina and Prieto, 1985).

Table 1.11.3.1 Protein digestibility (D) and protein efficiency ratio (PER) of some lupin seeds.

lupin species	pretreatment	D	PER	animal used
L. albus	+ met	nd	2.45	rat
L. albus	+ met, dehulled	nd	2.79	rat
L. albus	+ met	nd	2.45	rat
L. albus	+ met, lys, try	0.83	3.12	chick
L. albus	none	nd	0.13	rat
L. albus	none	nd	0.81	rat
L. albus	none	0.78	1.08	rat
L. albus	none	0.87	nd	pig
L. angust.	none	nd	1.30	rat
L. angust.	+ met	nd	3.36	rat
L. angust.	cooked + met	nd	3.58	rat
L. angust.	dehulled, + met, cook.	0.88	nd	rat
L. mutab.	none	nd	0.00	rat
L. mutab.	+ met	nd	1.56	rat
L. mutab.	+ met	nd	1.11	rat
L. mutab.	none	0.80	1.34	rat
L. mutab.	+ met	0.82	3.05	rat
soya	+ met, + lys	0.82	3.34	chick
soya	none	0.83	1.08	rat
soya	none	nd	1.59	rat
soya	+ met	nd	2.48	rat
casein	none	nd	2.96	rat
casein	none	nd	3.33	rat
casein	none	nd	3.09	rat
casein	+ met	nd	3.83	rat

Compiled from data by: Hove, 1974; Ruiz, White and Hove, 1977; Hove, King and Hill, 1978; Guillaume, Chenieux and Rideau, 1979; Schoeneberger, Gross, Cremer and Elmfada, 1982; Aguilera, Molina and Prieto, 1985; Aguilera, Prieto, Fonolla and Gil, 1986.

L. angust. : *Lupinus angustifolius*; *L. mutab.* : *Lupinus mutabilis*.

Lupin seed is an alternative good quality high protein and energy supplement for both cattle and sheep. Diets supplemented with lupin seeds give better growth rates (Carbon Arnold and Wallace, 1972; Hawthorne and Fromm, 1978; Kenney, 1981; Hawthorne, 1984). The AME of lupin seed for cattle is reported to be about 11.2 MJ kg^{-1} (Dawson, 1984).

Studies on protein digestibility in the rat, pig and poultry have shown that, when adequately supplemented with methionine and in some cases lysine, lupin protein has an excellent digestibility similar to that of casein (Ruiz, White and Hove, 1977; Ballester, Yanez, Garcia, Erazo, Lopez, Haardt, Cornejo, Lopez, Pokniak and Chichester, 1980; Schoeneberger, Gross, Cramer and Elmfada, 1982). The protein efficiency ratio (PER) for lupins has been shown to be dependant on the variety of lupin seed fed and on the pretreatment of the seed. The protein digestibility and PER for a number of lupin species are summarised in Table 1.11.3.1.

As can be seen (Table 1.11.3.1) in almost every case where lupin seed was supplemented with methionine the PER was as good as for casein. The notable exceptions were the two cultivars of L. *mutabilis* in which the high concentration of alkaloids present were attributed to be the causative factors (Hove, King and Hill, 1978). Similarly low results were reported by earlier workers using high alkaloid varieties of lupin (Tannous, Shadarevian and Cowan, 1968; Hill, 1977) however supplementation of this species did not raise the PER to the same level as casein even when the seeds were debittered. If the protein content of lupins is calculated by multiplying the nitrogen content by a factor of 5.7 instead of 6.25 because of the presence of non-protein nitrogen (Hudson, Fleetwood and Zand-Moghaddam, 1976; Wink, 1984) then the PER of lupins would be higher. Consequently the protein content of lupins would be considered as being lower and this may be considered to be undesirable by proponents of lupin seed as a high protein foodstuff. Some authors do, however, report protein contents of lupinseed as Nx5.7 (Aguilera and Trier, 1978). In the absence of methionine, protein quality of lupins is obviously poor. Recent work (Savage, Young and Hill, 1984) has demonstrated that methionine supplementation above levels of 3g kg^{-1} has no added effect on the biological value of lupin protein when it is the sole source of protein in diets for rats. Supplementary levels of methionine up to 4.2 g kg^{-1} diet containing lupins (230g kg^{-1}) has been shown to enhance the performance of pigs (Leibholz, 1984).

Work with pigs has shown that the lupin protein is of high quality and at least as good as soya protein particularly if supplemented with lysine and methionine (Taverner, 1975; Pearson and Carr, 1976; Pearson and Carr, 1979; Batterham, 1979; Barnett and Batterham, 1981; Aguilera, Molina and Prieto, 1985). In

general, reported nitrogen digestibilities of lupin protein by pigs is about 0.9 and is relatively independent of species and variety (Gladstones, 1970a; Taverner, Curic and Rayner, 1983). Similar levels of digestible nitrogen of lupins have been reported for other animals (Gladstones, 1970a; Hill, 1977).

For poultry, nitrogen digestibility has been reported to be slightly lower than that for pigs and there is slight lupin species dependancy (Hill, 1977; Guillaume, Chenieux and Rideau, 1979). Recent in-vitro studies on lupin protein degradability by trypsin has shown that protein degradation is partially dependant on the amount of carbohydrate attached to the protein (Semino, Restani and Cerletti, 1985). The increase in degradability of the protein on release of the carbohydrate was postulated to be caused by removal of steric hindrance caused by the bound carbohydrate. Perhaps in-vivo nitrogen digestibility is similarly restricted.

1.11.4 Antinutritive and toxic components of lupin seed

Lupin seeds are relatively free from deleterious components. The major components of lupin seeds which cause problems, particularly for bitter varieties, are the lupin alkaloids. These alkaloids are quinolizidine alkaloids (Fig. 1.8.7.1) and are found to vary in concentration within cultivars and between species (Table 1.11.4). There is reported to be considerable variation in alkaloid content between samples of lupins grown in various locations (Priddis and Harris, 1984). Capillary gas chromatographic analysis of samples of L. *angustifolius* grown at ten sites in Australia showed that the total alkaloid concentrtion varied between 0.02 to 0.84 g kg^{-1} (Priddis and Harris, 1984).

The major alkaloid components of the thirty or so found in lupins (Mears and Mabry, 1971; Wink, 1984) are angustifoline, a-isolupanine, lupanine, 13-hydroxylupanine and spartein and these account for up to 95% of the total alkaloids found in lupins (Priddis, 1983). The most common alkaloid found in the majority of lupin species is reported to be lupanine (Wink, 1984) but there is considerable variation in the relative proportions of lupanine and the other alkaloids, between lupin species (Ruiz, 1978; Kinghorn, Selim and Smolenski, 1980; Priddis, 1983; Wink, Witte, Hartmann, Theuring and Voltz, 1983; Wink, 1984; Wink and Witte, 1985). From the little work has been done on the relative toxicity of the various alkaloids it has been shown that there are considerable differences between them (Hatzold, Elmfada, Gross, Wink, Hartmann and Witte, 1983; Wink, 1983; Wink, 1985). The relative toxicity and bitterness of the component alkaloids in lupins may be highly significant if plant breeders are to improve the palatability of lupins for animals as well as for humans. Improvement in the palatability and nutritional quality has been demonstrated by exhaustive extraction (4-5 days) in water and sodium chloride solution

Table 1.11.4 Alkaloid content of lupin seeds used for animal feeds.

Species	alkaloid content* (g kg^{-1})	mean+ (g kg^{-1})
L. mutabilis	1.7-4.7	2.8 (8: 1.0)
L. albus	0.1-39.0	10.2 (16: 14.2)
L. leuteus	0-0.9	0.42 (6: 0.40)
L. angustifolius	0.02-6.5	1.27 (8: 2.22)

+ mean (number of samples: standard deviation)
* method of estimation varies.

For *L. albus* if 11 of the data points each having values less than 20 g kg^{-1} are only considered then the mean value is 1.39 (11: 1.78). The other 5 values can be considered as being obtained from bitter cultivars although one cultivar was reported as sweet but contained alkaloids at a level of 39g kg^{-1} (Eskin and Warenko, 1985). Sweet cultivars are considered to contain less than 20g alkaloid kg^{-1} seed (Aguilera and Trier, 1978).

Compiled from data in:
Tannous, Shadarevian and Cowan, 1968; Hove, 1974; Ruiz, White and Hove, 1977; Batterham, 1979; Guillaume, Chenieux and Rideau, 1979; Ballester, Yanez, Garcia, Erazo, Lopez, Haardt, Cornejo, Lopez, Pokniak and Chichester, 1980; Keller and Gross, 1980; Barnett and Batterham, 1981; muindi and Rundgren; 1981; Schoeneberger, Gross, Cremer and Elmfada, 1982; Haraszti and Vetter, 1983; Hatzold, Elmfada, Gross, Wink, Hartmann and Witte, 1983; Eskin and Warenko, 1985; Godfrey, Mercy, Emms and Payne, 1985; Aguilera, Prieto, Fonola and Gil, 1986.

(Schoeneberger, Gross, Cremer and Elmfada, 1982; Rhama and Rao, 1984). Such treatment also reduces the concentrations of other potentially antinutritive factors (Rhama and Rao, 1984).

Lupin alkaloids are reported (Keeler, 1973a; Keeler, 1973b; Aguilera and Trier, 1978; Keeler and Gross, 1980) to affect the central nervous system and include congenital malformations in calves, paralysis, stomach pains, cramps and death in humans. Angyrine is reported to be the alkaloid responsible for the teratogenic effects in calves born to cows fed on lupins during gestation (Keeler, 1973a; 1973b; Keeler and Gross, 1980).The alkaloids are also reported to be partially responsible for reduced growth and food intakes in animals (Wink, 1984; Godfrey, Mercy, Emms and Payne, 1985). They are also inhibitors of seed germination (Wink, 1983).

The other major component of lupin seeds which affect their nutritive value is the carbohydrate content which is primarily pectic in nature and most of which resides in the cotyledon cell walls (Brillouet and Riochet, 1983; Crawshaw and Ried, 1984; Brillouet, 1984; Carre, Brillouet and Thibault, 1985; Carre and Leclercq, 1985) rather than the seed coat (hulls). The cotyledon cell wall carbohydrates are composed mainly of galactose and mannose (674g kg^{-1} dry cotyledons) with small amounts of arabinose, glucose, uronic acids, xylose fucose and rhamnose (Crawshaw and Ried, 1984; Carre, Brillouet, Thibault, 1985). This is in contrast to the carbohydrate composition of the cotyledon of other legume seeds (Brillouet and Carre, 1983; Carre and Brillouet, 1986; Champ, Brillouet and Rouau, 1986). The cell wall material and thus the component carbohydrates from lupin cotyledons has been shown to be of little nutritional value to poultry (Carre and Leclercq, 1985). The major sugar component in the hulls is glucose which comes primarally from the cellulose present (Brillouet and Riochet, 1983). Treatment of hulls with cellulase and hemicellulase has hydrolysed about 80% of the carbohydrates (Bailley, Mills and Hove, 1974). Rats fed hulls from lupins had depressed food efficiency and growth rates (Bailley, Mills and Hove, 1974).

Stachyose has been shown to be the most abundant oligosaccharide present in a large variety of lupin seeds (Macrae and Zand-Moghaddam, 1978; Saini and Gladstones, 1986). The presence of stachyose in lupins will contribute to the flatulence effects caused by lupins.

Other potentially toxic and antinutritional factors in lupin seeds include lectins, trypsin inhibitors, saponins and polyphenolics. Both lectins and trypsin inhibitors are present in lupin seeds but the activity of these is very low (Table 1.8.1) and in some cases reported to be absent (Hill, 1977; Muzquiz, Vidal and Cassinello, 1984). Polyphenolics are also reported to be present in concentrations between 8 and 25g kg^{-1} (Haraszti and Vetter, 1983). Saponins have been detected and are

reported to be present in amounts varying between 7 and 29 g kg^{-1} (Hudson and El-Difrawi, 1979; Haraszti and Vetter, 1983; Muzquiz, Vidal and Cassinello, 1984). Small quantities of erucic acid has been reported to occur in lupins (Hudson, 1979). Although lupins belong to the Papilionoideae they are reported not to contain canavanine (Bell, Lackey and Polhill, 1978).

1.12 METHODS OF ANALYSIS OF CANAVANINE, CANALINE, MIMOSINE AND 3-HYDROXY-4(1H)-PYRIDONE.

1.12.1 Canavanine

Although canavanine contains the guanidino group it does not undergo reactions such as the Sakaguchi reaction. It is less basic than arginine having an isoelectric pH of 7.9 (arginine pH= 10.8) and is more stable in alkaline solution but less stable in acid solution than arginine (Rinderknecht, 1960; Greenstein and Winitz, 1961; Boyar and Marsh, 1982). Although lack of stability of canavanine in acid conditions has been reported, recent work has shown that canavanine is stable in 6M HCl at 100 deg. C for 48h (Rosenthal, Downum and Mattler, 1983).

The differences in chemistry can be exploited in order to assist in the separation of canavanine from arginine as well as the identification and analysis of canavanine (Boyar and Marsh, 1982). Canavanine reacts with light activated sodium nitrosopentacyanoferrate (III) (sodium nitroprusside) in neutral aqueous solution to yield a relatively stable pink or magenta colour (Kitagawa and Yamada, 1932; Rosenthal, 1977a; Natelson, 1985). This reaction with its resultant pink or magenta chromophore has been used as the basis for the most common method of detection and quantitation of canavanine. For the reaction to proceed it is necessary for the nitroprusside reagent to be in its oxidised [Fe(III)] state which is accomplished by addition of a variety of oxidising reagents and treatment with light (Kitagawa and Yamada, 1932; Fearon, 1946; Fearon and Bell, 1955; Rosenthal, 1977a). Under these conditions the active chromophoric molecule is reported to be pentacyanoaquoferrate (III) $[Fe(CN)_5OH_2]^{2-}$. However the presence of NH_3 in the reaction mixture produces pentacyanoammine-ferrate (III) $[Fe(CN)_5\ NH_3]^{2-}$ (PCAF) which is currently the reagent of preference since it can be readily obtained inexpensively as its reduced disodium salt (Fearon, 1946; Rosenthal, 1977a; Natelson, 1985). It is used after light activation in the presence of an oxidising reagent such as potassium persulphate. Colorimetric or spectophotometric methods (520-530nm) of detection and quantitation are used. (Rosenthal, 1977a; Rosenthal and Dahlman, 1982; Natelson, 1985). The working range of canavanine for detection by this method is between about 0.05 and 0.5 umols/cm^3 of solution (Rosenthal, 1977a).

A major disadvantage in using PCAF is its lack of specificity, associated with the pH dependency of the reaction conditions (Fearon, 1946; Rosenthal, 1977a; Natelson, 1985). In neutral conditions the reagent is reported to be specific for guanidinoxy compounds (Greenstein and Winitz, 1961) however this is patently not the case (Fearon, 1946; Bell, 1958; Birdsong, Alston and Turner, 1960; Rosenthal and Dahlman, 1982; Natelson, 1985). Other compounds produce chromophores with a shifted absorbance maximum (Fearon, 1946) but the histidine-PCAF complex has an absorption maximum which is close to that of the canavanine-PCAF complex (Rosenthal and Dahlman, 1982; Natelson, 1985). This particular point has led to some discussion in the literature regarding the suitability of PCAF for the estimation of canavanine (Rosenthal and Dahlman, 1982; Natelson, 1985). At pHs between 8 and 9 a large variety of compounds found in biological material reacts with PCAF producing red-purple-orange chromophores (Fearon, 1946). Animal physiological fluids are also reported to strongly inhibit the formation of the characteristic guanidinoxy-PCAF chromophore (Fearon and Bell, 1955).

In conjunction with chromatographic techniques such as paper chromatography (PC), paper electrophoresis (PEC), thin layer chromatography (TLC) and ion exchange chromatography (IEC) PCAF acts as a good method of identifying canavanine and is more selective than the general reagents (ninhydrin) commonly used for amino acid detection, so long as the pH is tightly controlled (Fearon and Bell, 1955; Bell, 1958; Birdsong, Alston and Turner, 1960; Turner and Harborne, 1967; Takahara, Nakanishi and Natelson, 1971; Rosenthal, 1974; 1977a; Rosenthal and Dahlman, 1982; Natelson, 1985). It is of note that PCAF also reacts with deaminocanavanine, a cyclised a-deamination product of canavanine, formed under mild conditions which can exist during extraction of canavanine (Bell, 1960; Rosenthal, 1972a). The deaminocanavanine-PCAF complex has an absorption maximum of 535nm. Under these circumstances spectrophotometric analysis of a mixture of canavanine and deaminocanavanine will overestimate the amount of canavanine present. Although PCAF has failings it has assisted in successfully disproving claims that canavinine exists in the fungus Agaricus campestris and in soya bean (Rosenthal and Davis, 1975; Rosenthal, 1978).

Using ion exchange chromatography, in conjunction with the other components of an amino acid analyser, it is possible to analyse canavanine independantly of interfering compounds (Rosenthal and Davis, 1975; Charlwood and Bell, 1977; Walker, D'Mello and Acamovic unpublished work). The method of detection frequently used in amino acid analysers is postcolumn reaction with ninhydrin to produce the blue violet colour which is easily and sensitively detected by colorimetry. Another method, but which is not so sensitive, is postcolumn reaction with 2,4,6-trinitrobenzene sulphonic acid in alkaline conditions to

produce the yellow Meisenheimer complex. This chromophore is also detected colorimetrically. Quantitative and reproducible results are obtainable at concentrations above about 1uM.

An enzymatic method capable of detection of canavanine, as the oxime of pyridoxal 5' phosphate and canaline, at similar sensitivities, has also been developed (Korpela, Lorenz and Laakso, 1982). The presence of canaline in the original extract is accounted for using a reference which has not been treated with arginase. The method is more specific than that using PCAF but requires overnight incubation with arginase. Canavanine measurement in leaves by this technique is not readily applicable because the green pigment which is simultaneously extracted from the plant absorbs strongly at the detection wavelength (Korpela, Lorenz and Laakso, 1982).

The most sensitive method for the quantitation of canavanine and its homologues is that of radiolabelling (Warren and Hunt, 1971; Rosenthal, Downum and Mattler, 1983), a technique which has been used to study canavanine metabolism in plants (Warren and Hunt, 1971; Rosenthal, 1982; Rosenthal and Rhodes, 1984). Radiolabelling techniques, although sensitive, are inconvenient, expensive and require highly specialised equipment such as gas chromatography (GC), mass spectrometry (MS) and scintillation counting equipment (Warren and Hunt, Rosenthal and Rhodes, 1984). These techniques do not readily lend themselves for routine analysis.

Recently a fluorometric method, involving some elegant chemistry, for the analysis of canavanine in plant material was developed (Natelson and Bratton, 1984, Natelson, 1985). The method is based on the fact that guanidines react with diketones in alkaline solution (Sakaguchi, Tanabe, Yagi, Miyakawaki and Saito, 1977). If the diketone is aromatic such as phenanthrene quinone (9,10-phenanthrenedione; PQ) then the resultant condensed ring product (2-amino-1H-phenanthro[9,10-d]-imidazole) is fluorescent (Baker, Mohammed and Veening, 1981). The reaction is specific for guanidino compounds (Baker, Mohammed and Veening, 1981; Natelson and Bratton, 1984; Natelson, 1985) and has been used for the detection of such compounds, other than canavanine, after separation by column ion exchange chromatography and reversed phase high performance chromatography (RPLC; Yamada and Itano, 1966; Yamamoto, Saito, Manji, Maeda and Ohta, 1979; Yamamoto, Manji, Saito, Maeda and Ohta, 1979; Baker, Mohammed and Veening, 1981).

Although canavanine and other guanidino compounds react with PQ it has been demonstrated (Natelson and Bratton, 1984) that guanidine gives the best fluorescent response, allowing detection at levels of 5-30 nmol cm^{-3}. The ready reduction of canavanine to homoserine and guanidine (Kalyankar, Ikawa and

Snell, 1958; Takahara, Nakanishi and Natelson, 1971; Natelson and Bratton, 1984; Natelson, 1985a) therefore permits the sensitive detection of canavanine after separation from other guanidines. It appears feasible that indirect analysis of canavanine is also possible by measurement of the homoserine produced during the reduction. The reduction of canavanine allows confirmatory analysis to be performed on guanidine mixtures such as extracts from JB, clover and Lucerne seeds (Natelson, 1985a).

The analysis of such mixtures can be achieved by semi automated ion exchange separation of the guanidino compounds followed by post column reaction with PQ (Natelson, 1985a). Levels of canavanine in the range 100-800nmol can be detected using this technique.

The PQ method suffers from high background fluorescence, potential losses during the fairly involved extraction procedures used, and the relatively lenghthy analysis time (about 100min). Precolumn derivatisation with PQ prior to chromatography of the derivatives may be a suitable alternative which could eliminate some of the disadvantages described above.

The use of HPLC (see sect. 1.13) has made rapid and routine amino acid determination at the femto mol (fmol) level with minimal interference. The amino acids can be analysed in less than 1h with pre- or postcolumn derivatisation. A number of derivatisation reagents can be used which allow fluorescence or ultraviolet (UV) detection. These include ninhydrin, orthophthaldialdehyde (OPA), fluorescamine, 5-N,N-dimethyl aminonaphthalene sulphonyl chloride (dansyl chloride), 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole and its chlorine analogue (NBD-F and NBD-C respectively), phenylisothiocyanate (PITC) and 9-fluorenylmethyl chloroformate (FMOC) as well as others (Imai, Toyo'oka and Miyano, 1984; Vendrell and Aviles, 1986). None of these reagents has all the desirable characteristics required for amino acid and amine analysis. The use, advantages and disadvantages of all these reagents for amino acid and amine analysis has been thoroughly reviewed recently (Imai, Toyo'oka and Miyano, 1984, Elkin, 1984; Brunton, 1986).

The use of OPA for the estimation of amino acids by post column reaction from dedicated amino acid analysers has been conducted for about 15 years (Roth and Hampai, 1973). OPA reacts with primary amines in the presence of 2-mercaptoethanol to produce highly fluorescent alkyl substituted isoindoles (Simons and Johnson, 1976).

The OPA adducts are formed rapidly and have a high quantum yield, therefore highly sensitive (Lindroth and Mopper), but undergo spontaneous intramolecular rearangement to yield non-fluorescent products (Simons and Johnson, 1976). If

ethanethiol is used instead of mercaptoethanol in the reaction, the adducts are more stable (Simons and Johnson; 1977).

Using HPLC and OPA/ethanethiol to produce the fluorophore, canavanine has been quantitatively determined, after exhaustive extraction, in tablets composed of Lucerne (Weissberger and Armstrong, 1984). Canavanine was derivatised prior to injection onto the column. Using this HPLC method, recovery and reproducibility was good and the analysis time was about 16 min. Detection was in the range 1-6 mM, which equates with on column levels of 20-120 nmol.

1.12.2 Canaline.

Although canaline is an important metabolite and precursor of canavanine there is only a single method available for its quantitative estimation (Rosenthal, 1973). The method depends on the formation of an orange chromophore when canaline reacts with 2,4,6-trinitrophenol (picric acid) at high pH. The resultant chromophore is then quantified spectrophotometrically at 490nm.

Linear responses were obtained with canaline contents of 0.1-0.8 umol (about 0.05-0.4mM). The advantage in using this technique for measuring canaline is that it is specific to canaline in the presence of canavanine and other amino acids. The major limitation is that the formation and decomposition of the chromophore is both pH and time dependant as well as being sensitive to the presence of other compounds such as creatinine (Rosenthal, 1973). There must be potential in analysing canaline by chromatographic technique followed by detection by reaction with reagents such as ninhydrin.

Qualitative methods for the analysis of canaline include paper chromatography folowed by ninhydrin spraying and also by autoradiography of radiolabelled canaline (Warren and Hunt, 1971). Electrophoresis has also been used to separate canaline, detection was by ninhydrin staining (Rahiala, 1973).

Although not designed to measure free canaline, the latter part of the enzymatic method used for the determination of canavanine in plant extracts by formation of the oxime between canaline and pyridoxal 5' phosphate followed by spectrophotometric detection, may be a suitable alternative method (Korpela, Lorenz and Laakso, 1982). A restriction in using such a method may be the low concentrations of canaline which occurs naturally. Another is the interference caused by plant pigments.

1.12.3 Mimosine

The well established method for the estimation of mimosine in plant extracts is by the colorimetric study (at 535nm) of the violet, octahedral tris-iron (III)

complex which is formed at low pH (Matsumoto and Sherman, 1951; Tsai and Ling, 1973). The chelation with Fe (III) is a characteristic reaction of phenols at low pH and is one method used for the determination of phenolics in plant materials (Burns, 1963; Fraser and D'Mello, 1981). Because of the interference caused by other plant phenolics preliminary treatment, normally with activated charcoal, is necessary (Matsumoto and Sherman, 1951; Megarrity, 1978). The addition of carbon to reduce the concentration of interfering compounds, however, also reduces the amount of measured mimosine present (Megarrity, 1978; Acamovic and D'Mello, 1981). This reduction is particularly significant when low levels of mimosine are being determined (Acamovic, unpublished observation; Lowry, Tangendjaja and Cook, 1985). The quantity of carbon used, and the relative amount of impurity present, has been shown to have a highly significant effect on the quantification of mimosine (Megarrity, 1978).

The presence of 3,4-DHP in the extract is a major limitation to using the Fe(III) chelation method for the determination of mimosine since 3,4-DHP forms similar complexes with Fe(III). The complexes of both compounds have similar spectral properties. The extinction coefficient is slightly lower for the 3,4-DHP complex but the absorption maxima are identical to each other and are therefore indistinguishable in colorimetric analysis. The result is that mimosine values determined using this method are normally higher than those determined by other methods (Acamovic and D'Mello, 1981; Lowry, Tangendjaja and Cook, 1985). In contrast, comparison of results obtained from a semi-automated method using Fe(III) and an AutoAnalyser system, with those from an electrophoretic method, gave closely correlated values (Megarrity, 1978). The determination of both mimosine and 3,4-DHP using the semi-automated technique and calling the result "mimosine" is considered satisfactory if the *leucaena* is to be used for ruminants since both compounds are regarded as toxic (Megarrity, personal communication). The technique has recently been used to assist in the development of low mimosine lines of *leucaena* (Hutton, 1985). Due to the problems associated with using a simple extraction procedure and Fe(III) as the detection reagent it may not be possible to accurately assess which lines are truelly low mimosine varieties. It is not possible to accurately assess the degradation of mimosine by this method, as has been attempted (Wood, Carter and Savory, 1983), because of the similarity in extinction coefficients (Megarrity, 1978) and identical absorption maxima of mimosine and 3,4-DHP chelates.

Since the pH of the chelation is an important factor in using Fe(III) this severely restricts the extraction technique used and which can be important in preserving mimosine (Hegarty, Court and Thorne, 1964; Wills and Tangendjaja, 1981; Acamovic and D'Mello, 1981; Lowry, Maryanto and Tangendjaja, 1983). Although there are inaccuracies and problems with the method it is used

extensively because of its simplicity and, in frequent cases, other techniques are not available.

If separation techniques are used prior to treatment of extracted mimosine with Fe(III) then the methodology becomes more complex but much more specific. A paper chromatographic method has been used to perform the separation of contaminating compounds in *leucaena* leaves and seeds as well as sheep urine subsequent to sample preparation by ion exchange chromatography (Hegarty, Court and Thorne, 1964). After chromatography the component spots were identified by spraying with Fe(III). The spots were then eluted from the paper and spectrophotometrically quantified using Fe(III). Good recovery of 10-160ug of mimosine was obtained. Chromatography took 15h and the colour was found to fade in light. Although these disadvantages exist this method has been successfully used recently to determine mimosine in animal and plant tissues (ter Muelen, Pucher, Szyszka and El-Harith, 1984) and to monitor the enzymic degradation of mimosine in *leucaena* (Lowry, Maryanto and Tangendjaja, 1983). The presence of iron in physiological fluids has been shown to affect paper chromatographic estimations of mimosine (Tsai and Ling, 1974). Removal of iron by alkaline precipitation prior to chromatography solved the problem.

TLC has also been succesfully used for the separation and quantitation of mimosine and its metabolites from *leucaena* and physiological fluids (Librojo and Hathcock, 1974; Alejandrino, Goze and Balboa, 1976) using Fe(III) and ninhydrin as detection agents. Using both detection methods allowed ready identification of phenolic amino acids. Detection of mimosine in soya sauce extract, with Fe(III) after separation by IEC, has also been achieved (Cheng and Ling, 1968). Mimosine in sheep urine has been determined using electrophoresis with spectophotometric detection of the Fe(III) chelate (Reis, Tunks and Hegarty, 1975; Megarrity, 1978). The method is reported to work well for mimosine in the range 1-5ug of mimosine.

Another technique which has been used for mimosine determination is GC (Mee and Brooks, 1971). The N-trifluoracetyl butyl ester of mimosine was prepared prior to chromatography and analysis of mimosine was achieved in about 7 min. A problem which occurs when using GC is the necessity to esterify the free mimosine. The conditions of the esterification procedure may tend to degrade mimosine while the diacetyl derivative is formed as well as the monoacetyl derivative. Thus sensitive control of the derivatisation procedure is imperative while quantitation is difficult.

Mimosine has also been analysed using amino acid analysers (IEC) although special buffer systems are necessary in order to avoid coelution of mimosine with other proteinaceous amino acids (Reis, Tunks and Chapman, 1975; Mzik, 1977;

Charlewood and Bell, 1977; Acamovic and D'Mello, 1981; Evans and Telek, 1982). These methods, except one (Acamovic and D'Mello, 1981), all utilise the conventional reaction between amino acids and ninhydrin to detect mimosine in physiological fluids as well as *leucaena* extracts. The other method utilises the reaction between amino acids and TNBS. Analysis times using IEC are of the order of 60min. These methods are unsuitable for the detection of 3,4-DHP unless Fe(III) is used as the post column reaction compound.

The techniques used for the estimation of mimosine, and discussed above have one or more limitations. These include lack of specificity, lengthy analysis time, difficulty in quantifying mimosine and, in some cases, constraints are placed on extraction procedures.

The use of HPLC for the analysis of mimosine, and some of its degradation products and derivatives, provides a rapid, sensitive and accurate method of analysis in a wide range of biological materials including excreta, urine and *leucaena* (Tangendjaja and Wills, 1980; Tangendjaja and Wills, 1983; Lyon, 1985; Lowry, Tangendjaja and Cook, 1985). Reversed phase HPLC was the method of choice in all but two cases, where ion exchange was used (Lowry, Maryanto and Tangendjaja, 1983; Lowry, Tangendjaja and Cook, 1985). Using orthophosphoric acid as the ion pairing agent mimosine, 3,4-DHP and 3,4-DHP glucuronide have been separated and quantified, with minimal interference, in less than 7 min (Tangendjaja and Wills, 1980; 1983). The method has also been used to determine mimosine in *leucaena* silage (Lyon, 1985).

Detection levels for analysis using IE HPLC was in the pico mol (pmol) range (Lowry, Tangendjaja and Cook, 1985). The sensitivity of this method is extremely high for compounds having an extinction coefficient at 282nm of 16,982cm^2 mol^{-1} (Merck Index) or less and are close to the detection limits. In each case, for use of the above HPLC methods, analysis was completed within 10min using UV detection where 3,4-DHP was the slower eluting peak.

The major advantages in using HPLC are that mimosine, DHP and their metabolites can be assayed rapidly and specifically at the same time without derivatisation.

1.12.4 3-hydroxy-4(1H)-pyridone

The analysis of 3,4-DHP has assumed importance in recent years since its toxicity became known. Methods of analysis of 3,4-DHP are based on those described above. The most commonly used method has been paper chromatography associated with Fe(III) detection (Hegarty, Court and Thorne, 1964). A semi automated method using AutoAnalyser technology has been

developed (Megarrity, 1981) however this method does not distinguish mimosine from 3,4-DHP or other phenolic compounds. The best methods developed for the estimation of 3,4-DHP are HPLC based methods. Such methods are identical to those described above for the analysis of mimosine.

1.13 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The use of HPLC for the analysis of many compounds is now commonplace and HPLC is now one of the major analytical techniques currently used. Its development occurred in the late 1960s. There are many books on both fundamental and advanced aspects of HPLC (Snyder and Kirkland, 1979; Pryde and Gilbert, 1979; Knox, 1982). Reviews and literature on specific aspects of HPLC also abound (Horvath, 1980a; 1980b; 1983; Hearn, 1985; Ahuja, 1986) while reviews of the most recent advances also exist (Barth, Barber, Lochmuller, Majors and Regnier, 1986). A brief summary of some aspects of the technique will be presented here.

HPLC is a complementary analytical technique to GC. The major differences are that GC is used for the analysis of compounds which are naturally volatile or which can be made to become volatile while HPLC is used for the analysis of non volatile compounds. The eluent for GC is a gas while that for HPLC is a liquid. The consequences of using a liquid eluent instead of a gaseous one are, that due to the increased viscosities of liquids (20 to 100 fold increase) the operating pressures are correspondingly higher. Rates of diffusion are 3000 to 30000 times lower in liquids than in gases thus the particle sizes require to be 50 to 200 times smaller than for GC (Knox; 1982). Particle sizes in current use vary in the range 3-10um and working pressures in the range 50 to about 350 bar (700-5000psi) at eluent flow rates of between 0.5 and 4 cm^3 min^{-1}. Target analysis times are usually in the range 5 to 60min and can be conducted isocratically or by gradient elution. The requirements for a system are almost identical to those for GC: pumps, injector, column, detector and data handling.

Packing materials for HPLC are of two general types; reversed phase and normal phase. Both types can include bonded phases where the primary functional moiety is covalently bound to a silica support. Normal phase packing material is usually bare silica and the eluents used are usually non polar alkanes. The most frequently used packing material is undoubtedly bonded reverse phase and probably those containing C_{18} (octadecyl) bound moieties (Majors, 1980). It is of note that packing materials which have the same functionality can vary considerably in their selectivity (Goldberg, 1982). Other more novel packing materials are available which are chemically more stable than silica based materials and include polystyrene/divinylbenzene copolymers (Benson and Woo, 1984) and graphitic carbon (Knox, Unger and Mueller; 1983). Analysis using

$$Rs = 1/2\{(1-a)/(1+a)\}\{\overline{k}'/(1+\overline{k}')\}\sqrt{N}\ e1/2$$

relative partition factor	degree of retention factor	column efficiency factor

$a = k'2/k'1$: selectivity

$\overline{k}' = 1/2(k'1 + k'2)$ hwere k'1 and k'2 are the column capacity factors of the two components with resolution Rs.

reversed phase packing materials usually requires the use of aqueous mobile phases containing an organic modifier, and probably buffered if the analytes are biological.

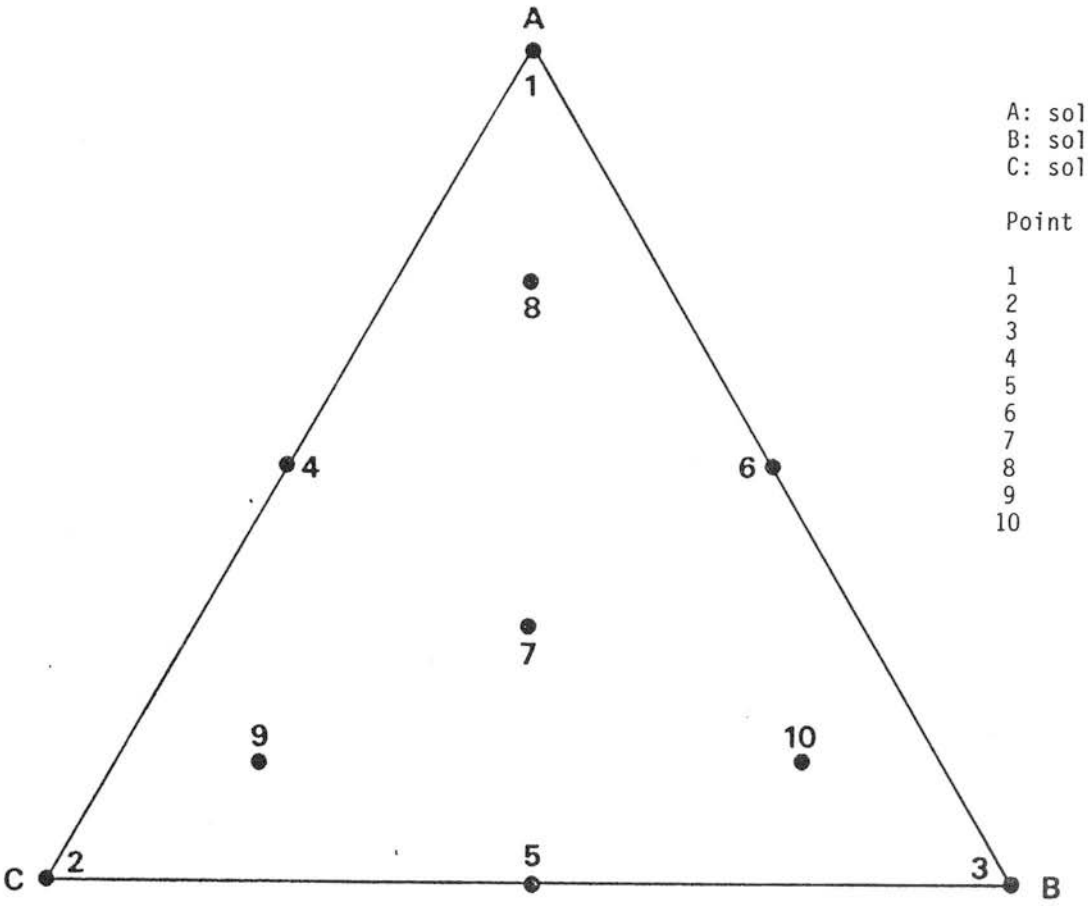
High efficiency analytical columns usually contain greater than 100,000 theoretical plates per meter (ie. N=100,000). Calculation of efficiency is done using a variety of methods each having advantages and disadvantages depending on peak shape (Bidlingmeyer and Warren, 1984). One of the simpler methods in determining column efficiency is to relate peak width at half height to retention time in the following fashion; $N = 5.54(tr/W1/2)x1000/L$ where tr = retention time, W1/2 = peak width at half height in the same units, L=column length. For comparison of column efficiencies it is important that the analytes used and the analysis conditions are standardised and simple. Efficiency is dependend on the dead volume of the system and this assumes greater importance as the bore (normally between 4 and 5 mm) decreases. It is therefore imperative that dead volumes in HPLC be minimised. The efficiency is also dependant on various other parameters such as particle diameter (dp) of the packing material, flow rate and various solvent characteristics (Snyder and Kirkland, 1979; Knox, 1982). The implications and effects of these have all been thoroughly discussed in the literature (Knox; 1977; Snyder and Kirkland, 1979).

The main aim in HPLC is to separate components in a mixture. The resolution (Rs) of these components is related to the selectivity, retentitivity and column efficiency. The following equation shows the relationship between Rs and these parameters:

The significance of these relationships have been fully explained (Knox, 1977; Snyder and Kirkland, 1979; Knox, 1982). The important points are that Rs is directly proportional to the square root of N (ie. double N and Rs is increased by a factor of 1.41) and that selectivity must be different for each component for resolution to occur. Selectivity is strongly influenced by eluent, which is analogous to temperature effects in GC. The composition of the solvent can be readily changed thus affecting the k' values which has the largest effect on Rs with minimal effort. Examination of the equation shows that k' values between 1 and 10 are optimal, having a larger effect on Rs than k' values greater than 10.

Variation of solvent mixtures is the technique most frequently used in order to obtain separation of a mixture of compounds. This can be time consuming and tedious. The procedure is helped if a knowledge of the solvent characteristics is available. Snyder (Snyder, 1978) has devised solvent strength and selectivity parameters based on their dipole, hydrogen bonding and dielectric properties. From this data a selectivity triangle was constructed (Snyder, 1978; Snyder and Kirkland, 1979) which readily shows the similarity or dissimilarity of groups of

Fig. 1.13.1 Simplex design for optimisation using three solvents.



A: solvent A
B: solvent B
C: solvent C

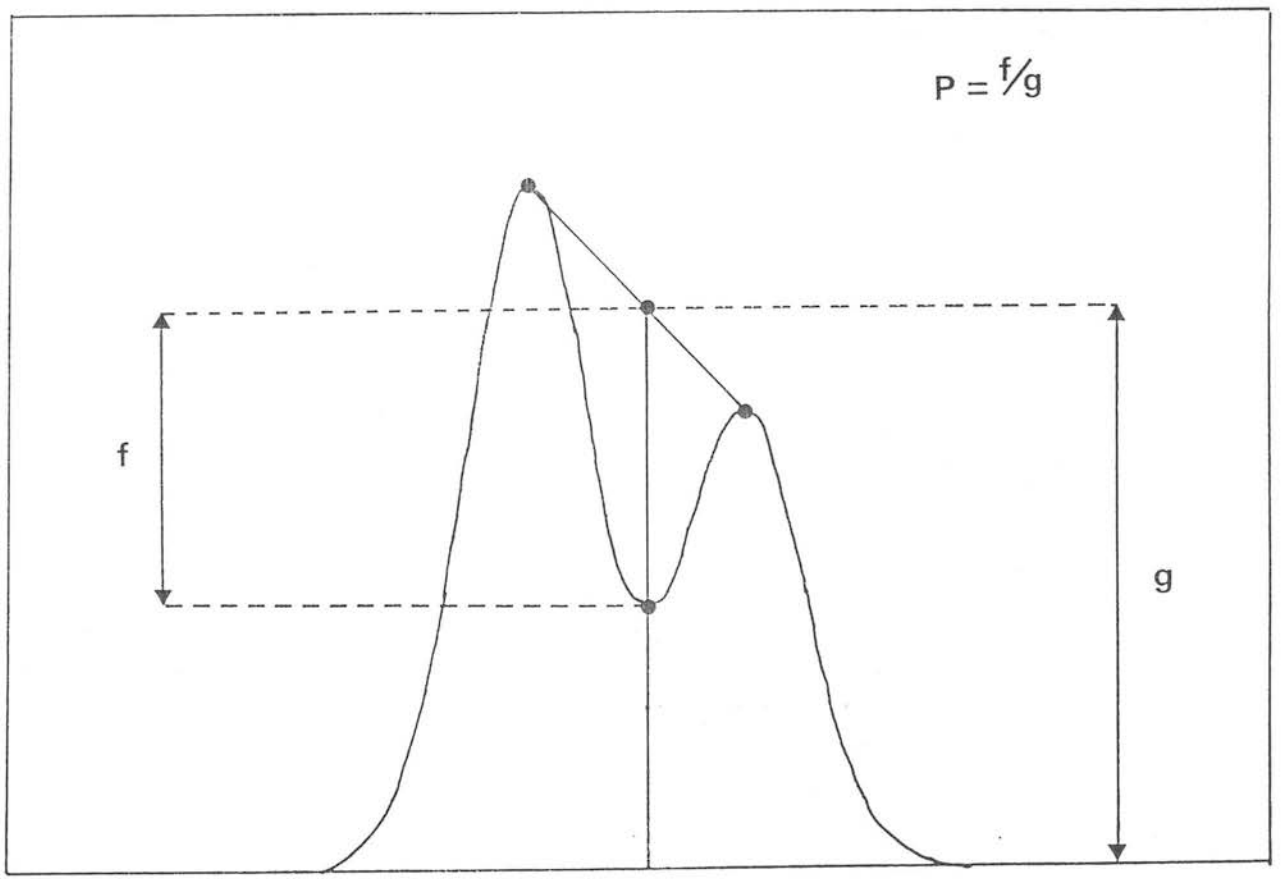
Point	A	B	C
1	1	0	0
2	0	0	1
3	0	1	0
4	0.5	0	0.5
5	0	0.5	0.5
6	0.5	0.5	0
7	0.33	0.33	0.33
8	0.67	0.16	0.16
9	0.16	0.16	0.67
10	0.16	0.67	0.16

Table 1.13.1 Solvent strength (S) for various solvents used in reversed phase HPLC.

solvent	S
water	0.0
methanol	3.0
acetonitrile	3.1
acetone	3.4
dioxan	3.5
ethanol	3.6
propan-1-ol	4.2
tetrahydrofuran	4.4

Data from Snyder and Kirkland, 1979

Fig. 1.13.3 Chromatographic peak separation factor P.



solvents. Selection of solvents at the apices of the triangle (ie. maximum differences in selectivity; Fig. 1.13.1) allows greatest variation in selectivity to be achieved in mixtures. The most common organic solvents used for reversed phase HPLC (RPLC) are methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF) which have a number of desireable properties for HPLC solvents (Snyder and Kirkland, 1979). The solvent strengths (S), for RPLC, of these and other solvents are shown in Table 1.13.1.

Solvent mixtures with equivalent strengths (but different selectivities) are then calculated relative to the respective proportions in that mixture. For example if the original eluent is composed of a mixture of methanol and water ($50cm^3$ + $50cm^3$) and a solvent comprising ACN and water of the same strength is required, it is obtained using the following calculation:

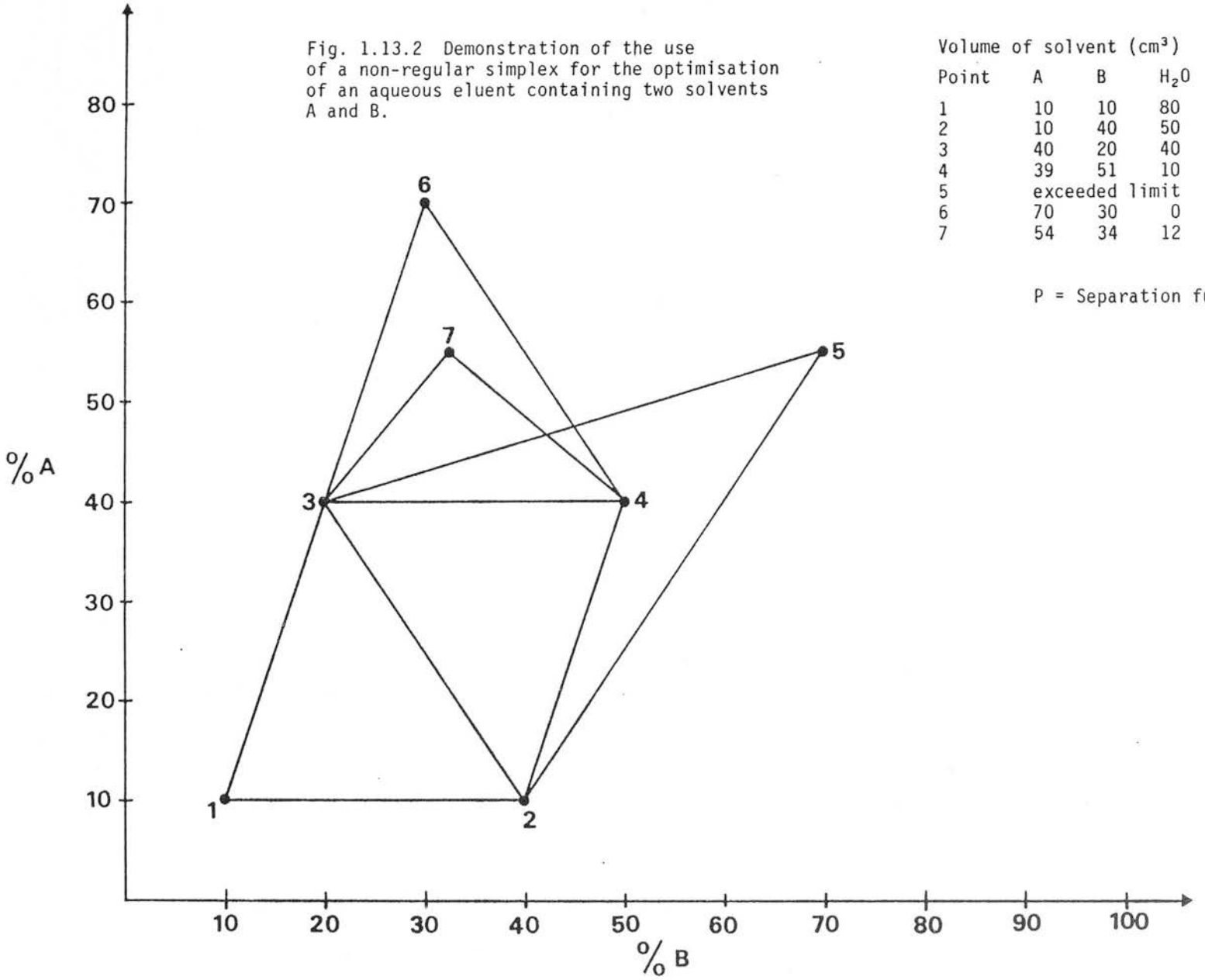
$Pc = Pb(Sb/Sc) = 0.5(3.0/3.1) = 0.48$

ie. the new solvent with the same strength comprises $48cm^3$ of ACN and $52cm^3$ water.

The significance of maintaining constant solvent strength allows method development without the disadvantage of vast changes in k' values. This method can be extended in order to help in development and optimisation of conditions by using a statistically based method (Glajch, Kirkland, Squire and Minor, 1980). If the eluent mixtures at the apices of the triangle have equal strength, then method development occurs using solvent mixtures with approximately equal strength. Equality of solvent strength is not a prerequisite for using the method, however it avoids excessively large k' values which are undesireable (Fig. 1.13.1). Method development using statistically based techniques involves less work and time compared to empirical methods.

Runs are conducted starting with eluent A on the triangle, then B etc around the triangle until the tenth chromatogram is obtained (point 10: composition is 0.16/0.67/0.16 A,B,C respectively). Visual examination of the chromatograms produced gives an indication of which is the best solvent mix. The resolution between peaks can be measured by a variety of methods (eg. distance between peak tops and valley; Fig. 1.13.3) and weighted depending on the relative importance of resolution between the peaks (Glajch, Kirkland, Squire and Minor, 1980; Cela and Perez-Bustamente, 1983; Nickel and Demming, 1983; Berridge and Morrisey, 1984; D'Agostini, Castagnetta, Mitchell and O'Hare, 1985). The weighted resolution factors can then be used to quantify which solvent mixture gives the best resolution in the shortest time. Quantification of resolution is necessary if computer methods are used for automated optimisation of the chromatography (Berridge, 1984; 1985).

Fig. 1.13.2 Demonstration of the use of a non-regular simplex for the optimisation of an aqueous eluent containing two solvents A and B.



Volume of solvent (cm^3)

Point	A	B	H_2O	P
1	10	10	80	-
2	10	40	50	0.3
3	40	20	40	0.4
4	39	51	10	0.6
5	exceeded limit			-
6	70	30	0	0.3
7	54	34	12	0.8

P = Separation functions

Another approach to method development which can be used independantly or in conjunction with the above method, also utilises simplex optimisation techniques of a different type (Morgan and Deming, 1975; Deming and Parker, 1978). This simplex methodology can be used to optimise the conditions or parameters in many systems. The number of parameters can vary and be upwards of two. Although the technique is extremely powerful if the parameters which vary exceed two, the technique becomes more difficult to use manually and requires a computer. A regular simplex may be defined as a set of (n+1) mutually equidistent points in n-dimensional space (Bunday, 1984). This means that in two dimensions the simplex is an equilateral triangle while a three dimensional simplex is a tetrahedron. In order to make this technique more powerful and allow the true optimum to be found the simplex contains three basic operations; reflection, expansion and contraction (Bunday, 1984). The use of the technique for an eluent containing two solvents and water is demonstrated in Fig. 1.13.2.

Points 1, 2 and 3 are selected arbitrarily but with some knowledgeable guess work, and the chromatography conducted. Since the resolution, measured simply as the peak separation (P; Fig. 1.13.3) of the two analytes of interest, is poorest for point 1 the triangle is expanded through the centre to yield point 5. This point is outside the boundary limit of a total of $100cm^3$ therefore is rejected. The point then taken is 4 which gives the regular simplex 2,3,4. Since the eluent composition at point 2 produces the worst resolution (ie. lowest P) of the three, reflection from point 2 yields 6 which exceeds the boundary limit. If point 6 was inside the boundary limit and P was poorer than for either 3 or 4 then a contraction to 7 is the next point. The contraction expansion and reflections are continued until P no longer improves (ie. approaches 1). The use of this technique, and modifications of it, and its advantages and disadvantages have recently been thoroughly reviewed (Berridge, 1985).

Further information on optimisation procedures and the different algorithms used are well described in the literature (Morgan and Deming, 1975; Deming and Parker, 1978; Nickel and Deming, 1983; Berridge, 1984; Berridge and Morrissey; 1984; D'Agostini, Castagnetta, Mitchell and O'Hare, 1985).

One of the problems with using such optimisation techniques described above is that peak elution order can change. This is not of major importance if the total number of peaks are known and the best possible resolution is required. However once the method is optimised each peak must be identified.

Detection systems in HPLC require to have low dead volumes therefore cell volume consisting of less than 10ul are normally used. The most frequently used detection systems rely on UV absorption and are most commonly used above about 230nm for the detection of aromatic compounds. If lower wavelength

detection is required special precautions must be taken in order to minimise the absorption caused by the eluent. It may be necessary in some cases to produce derivatives with UV absorbing chromophores so that detection is made easier and more sensitive. Another frequently used detector is the refractometer. It is the most general of all the detection systems used in HPLC, relying on refractive index changes caused by elution of the analytes. This type of detector is the least sensitive of those mentioned here and is inherently the most susceptible to environmental changes such as temperature. It is used frequently for the determination of carbohydrates (Macrae and Zand-Moghaddam, 1978; Saini and Gladstones, 1986). Another more sensitive and specific detector in general use is the fluorescence detector. This detector is used for the sensitive detection of compounds which fluoresce naturally after excitation by UV radiation, and for compounds which after derivatisation, fluoresce. Fluorescence detection is used routinely for the quantification of amino acids. The detector of highest sensitivity and specificity mentioned here is the electrochemical detector (ECD). The detector is used for analysis of compounds which are readily oxidised or reduced when a potential difference is applied. Compounds such as quinones are ideal candidates for detection by reduction while the reduced quinones; phenols are ideal analytes for oxidation. Since the oxidation and reduction of these compounds is achieved electrochemically eluents must necessarily contain relatively low concentrations of organic modifiers. Analysis of biogenic amines is one of the most significant uses of ECD. Both ECD and refractometers are highly susceptible to solvent composition changes and are therefore generally used for isocratic separations.

Further information on the detection systems and their uses and limitations can be obtained from the list of references given at the beginning of this section.

1.14 Conclusions from the review of literature

From publised work it is obvious that little attention has been given to the evaluation of LLM as a nutrient source for broilers. There is also a deficit of information on the quantity, variety and the deleterious effects of antinutritional components in LLM, other than mimosine.

The majority of workers who have studied methods of reducing the toxicity of mimosine in *Leucaena* have utilised Fe(II) salts and have tended to neglect the efficacy of other salts such as those containing Fe(III). The use of Fe(III) containing salts appears to have some potential in reducing the adverse effects of phenolic compounds because Fe(III) has better chelating properties with mimosine and other phenols than Fe(II) containing salts. No attempt has been made to reduce the adverse effects of the tannins and other antinutritional

components in LLM while attempts at increasing the AME of LLM by treatment with enzymes have been unsuccessful.

Based on the literature there is ample scope for assessing the effects of supplementing diets containing LLM with Fe(III) which has considerable affinity for mimosine and other phenolics thus reducing their ability to interfere in the digestive tract of poultry. Similarly, the addition of PEG4000 is expected to reduce the adverse effects of tannins while cholesterol may reduce the adverse effects of any saponins present in LLM. Heating may reduce the effects of any heat labile antinutrients as well as enhance the nutritional quality of the carbohydrates.

Methods available for the estimation of momosine and 3,4-DHP have suffered from various defects including lack of specificity, long analysis times and lack of sensitivity. As a consequence of poor analytical techniques for the analysis of mimosine and 3,4-DHP, the ability to monitor the fate of these compounds has been severely restricted and as a result, little information is available on the fate of mimosine and 3,4-DHP when consumed by poultry. Minimal information is available on the consequences of the consumption of mimosine by poultry in the absence of LLM.

There is even less information available on the toxic and nutritional status of JB for broilers than there is for LLM. Most of the early work on JB centred around the antinutritional effects of the haemagglutinins present and the effectiveness of heat treatment in reducing the effect of these. There is ample scope for studying the effect and fate of canavanine when consumed by poultry either as part of JB diets or when included separately into conventional soya/maize diets. The literature is almost devoid of reports on the consequences of the consumption of canavanine and/or heat treated JB by poultry and therefore similarly devoid of report of attempts to reduce the adverse effects caused by the consumption of JB or canavanine.

Since canavanine is an analogue of arginine and is likely to compete with arginine in the urea cycle (Fig. 1.10.4). It is feasible that supplementation of diets which contain JB with arginine may reverse or reduce the adverse effects of canavanine, by competing with canavanine in the appropriate degradation reactions *in-vivo*. The incorporation of canavanine into proteins with a consequential reduction or loss of functionality may be limited by the inclusion of supplemental arginine in the diet. In a similar fashion, incorporation of other urea cycle intermediates such as ornithine, may cause beneficial responses. On the other hand the combination of canavanine and arginine in the JB diet may cause an apparent imbalance of dietary guanidino amino acids with respect to the concentration of lysine in the diet. Under these circumstances lysine would

be a necessary dietary additive in order to balance the combined effect of dietary canavanine and arginine.

In a similar manner to LLM and mimosine, conventional methods of analysis severely restrict the analysis and monitoring of canavanine and its metabolites. There is therefore considerable scope for the development of a sufficiently rapid, sensitive and specific method for the analysis of these compounds. Similarly to the analysis of mimosine and its metabolites, HPLC holds great potential in providing a suitable technique for the analysis of canavanine and its metabolites.

Lupins have been studied fairly thoroughly with respect to the nutritional value of various cultivars of these for poultry particularly in Eastern Europe and the Antipodes. A considerable body of literature also exists on the carbohydrate and protein content and the composition of these in lupins.

Little work has been reported on the nutritional value of lupins grown in the United Kingdom for poultry and no work has been reported on the possibility of increasing the nutritional value of lupins for poultry by treatment with enzymes. B-glucanase treatment of barley has been successful in improving the nutritional value of barley for poultry while guar gums have been nutritionally improved by treatment with suitable enzymes. Similar responses may be obtained by treatment of lupins with polymeric carbohydrate degrading enzymes such as pectinase and a-amylase, so that digestible carbohydrates are produced.

CHAPTER 2

EXPERIMENTAL

2.1 General objectives.

There were two main aspects to this study. One of these was to develop reliable, accurate, specific, rapid and precise methods of analysis of mimosine, 3,4-DHP, canavanine and canaline in the biological matter of plants and in the animals which consumed them. With such methods it would be possible to monitor the fate of these compounds before, during and after ingestion. The other aspect of the work presented here was to assess and attempt to reduce the adverse effects of feeding LLM, JB and lupins to young chicks.

2.2 Analysis of diets, ingredients and excreta samples.

Proximate analysis was conducted using the methodology employed by the Central Analytical-Trace Elements Laboratory in the Edinburgh School of Agriculture and are expressed on a dry matter basis.

Dry matter was determined at 100°C while ash was determined on 2g samples at 500-550°C. Nitrogen was determined semi-automatically using a discrete analysis system for the colorimetric quantitation of the nitrogen present in the sulphuric acid/hydrogen peroxide digestate of 0.5g of sample (Crooke and Simpson, 1971). Modified acid detergent fibre (MADF) was determined gravimetrically according to the method of the Agricultural Development and Advisory Service of the Ministry of Agriculture, Fisheries and Food (ADAS, 1981). Trichloro-acetic acid fibre (TCA-fibre) was determined using a slight modification of the gravimetric method (Whitehouse, Zarow and Shay, 1945). Fat was determined by extraction of the sample with petroleum ether (bp. 40-60°C and weighing the extracted lipid.

Gross energy of the air dry samples was determined using adiabatic bomb calorimetry.

Elemental mineral analysis was conducted on the nitric/perchloric acid digests of the material. Sodium and potassium were quantified using atomic emission techniques while other elements were determined using atomic absorption.

Amino acid analyses were obtained by ion exchange chromatography of the 6M HCl hydrolysates of the samples as described elsewhere (D'Mello, 1972).

2.3 Analysis of mimosine and 3-hydroxy-4(1H)-pyridone.

The inability of most traditional techniques to determine both mimosine and 3,4-DHP quantitatively led to the development of an HPLC method using UV detection. The criteria necessary for such a technique was that it should be rapid, specific, sensitive and sufficiently robust as to be able to quantitate the analytes from a variety of matrixes; plant material, excreta and serum.

Another necessary requirement in such analysis is the use of standard compounds. During the initial stages of the method development of the HPLC method a 6h hydrolysate of mimosine was used. This mixture of mimosine and 3,4-DHP (Hegarty, Court and Thorne, 1964) allowed eluent and column choice to be made but did not allow quantification.

2.3.1 Preparation of standards.

Mimosine (extracted from *leucaena* seeds) was purchased (Sigma Chemical Company, London, U.K.) and a stock standard solution (0.25mM) was prepared by dissolving the appropriate quantity of oven-dried mimosine in 0.1M HCl. Working standards were prepared by diluting appropriate aliquots with HCl (0.1M). All standard solutions were stored at -20°C until required for use.

Since 3,4-DHP is not available commercially it was prepared from purchased mimosine by acid hydrolysis. The degradation is reported to occur yielding pyruvic acid and alanine as by-products (Hart, Hofmann, Lambert and Richards, 1977). Alanine has not been detected in some hydrolysates of mimosine (Mee and Brooks, 1971; Acamovic and D'Mello, 1981).

Mimosine is soluble and stable in 6M HCl but is readily degraded in hot 0.1M HCl (Hegarty, Court and Thorne, 1964; Acamovic and D'Mello, 1981). Mimosine (2g) was refluxed with 200cm^3 of 0.1M HCl for 24h. Ion exchange chromatography (Acamovic and D'Mello, 1981) of the resultant hydrolysate did not show the presence of any residual mimosine nor was any alanine detected. Purification of 3,4-DHP was achieved by preparative ion exchange chromatography (Hegarty, Court and Thorne, 1964). Using this technique, however, the 3,4-DHP could not be removed from the resin even after washing with 2.5dm^3 of 0.1M HCl. Elution of 3,4-DHP was achieved with 1M HCl (700cm^3). Washing the column with a further 500cm^3 of acid did not elute further Fe(III) positive material. The liquor containing the 3,4-DHP was

evaporated off using thin film rotary evaporation and the residue dissolved in about 15cm^3 of 0.5M acetic acid.

Dehydrochlorination of the prepared 3,4-DHP was achieved by passing the solution through a column (1.3 x 6cm) of anion exchange resin in the acetate form (Hirs, Moore and Stein, 1954). The acetate form of the resin was prepared from Amberlite resin in the chloride form (Amberlite IRA-400(Cl); obtained from: BDH Chemicals, Poole, U.K.). Conversion to the acetate form was accomplished by treatment of the resin with 1M NaOH (20 volumes : 1 volume of resin) until the washings were free from Cl anions. The resin was subsequently washed with deionised water until the pH was less than 9 then washed with 1M acetic acid until the pH of the eluent was less than 2. Finally the resin was washed with deionised water until the pH was greater than 4.8.

The 3,4-DHP solution was passed through the column eluting with 0.5M acetic acid under reduced pressure. The resultant solution was treated as described elsewhere (Hegarty, Court and Thorne, 1964) although vacuum sublimation of the 3,4-DHP was not performed. Repeated recrystallisation of 3,4-DHP from ethanol followed by washing with diethyl ether, yielded buff-coloured crystals which were placed in a desiccator and left for 24h under vacuum (m.p. 240-243; lit. 242-244 dec; Hegarty, Court and Thorne, 1964; Hart, Hofmann, Lambert and Richards, 1977). Infrared spectroscopy in nujol produced a spectrum consistent with that expected for 3,4-DHP while chromatographic analysis did not reveal other contaminants. The prepared 3,4-DHP reacted as expected with Fe(III) in 0.1M HCl giving the characteristic magenta colour. The UV spectrum showed a lambda max of 269nm and an extinction coefficient, in 0.1M HCl, of 516 m^2 mol^{-1} at 269nm. The product did not react with ninhydrin and had a nitrogen content, measured using a nitrogen analyser, of 125g kg^{-1}. The calculated nitrogen content for $C_5H_5O_2N$ is 126g kg^{-1} ie. 0.992 of the nitrogen expected was recovered. The slight discrepancy in purity compared with the previously published data may be because vacuum sublimation was not used in the final stages of preparation.

Standard solutions of 3,4-DHP in 0.1M HCl were prepared as described above for mimosine.

2.3.2 Sample preparation.

The method of preparation of finely ground LLM, *leucaena* seeds, freeze dried excreta and fresh excreta (about 20g of a homogenous mixture of fresh excreta and water) for the determination of mimosine and 3,4-DHP has been described

elsewhere (Acamovic and D'Mello, 1981). An added step was that the extract was forced through a Sep-Pak C18 cartridge (Millipore (UK) Limited, Harrow, U.K.). Washing the Sep-Pak with 0.1M HCl (2 x $5cm^3$), methanol (2 x $5cm^3$), distilled H_2O (2 x $5cm^3$) followed by 2-$3cm^3$ of extract, prior to collection of the eluate form the Sep-Pak, produced cleaner samples for chromatography. This preparative procedure for the Sep-Pak allowed reuse of a cartridge for about 20 samples. Chick serum was prepared by allowing whole chick blood to stand overnight at 4°C prior to centrifugation at 2500g for 8 min. the resultant serum was decanted off and stored at -20°C until required for analysis.

Protein precipitation in the serum was accomplished using two procedures. One method involved the use of sulphosalicylic acid (SSA; 8g in $100cm^3$ H_2O) while the other utilised the properties of phosphotungstic acid (PTA; $H_3PO_4.12WO_3.xH_2O$; 6g in $100cm^3$ H_2O). Precipitation of the protein was accomplished by adding 0.25 cm^3 of either of the precipitant solutions to $1cm^3$ of serum and centrifuging (3000g) for 5 min. The supernatent was decanted off and passed through a pretreated Sep-Pak as described above. In this case the cartridges were discarded. At this stage the deproteinised serum was ready for chromatography.

Initial attempts to precipitate the protein from serum involved the use of saturated ammonium sulphate and ethanol, however addition of SSA caused further precipitation of protein and therefore the use of these reagents was discontinued.

2.3.3 Recovery of mimosine and 3,4-DHP from samples

The recovery of supplemental mimosine from LLM using the extraction technique previously reported (Acamovic and D'Mello, 1981), has been determined using IEC. Similar studies with dried chick excreta gave recoveries of 102.7%. Since recoveries of mimosine were quantitative the IEC technique was used as the reference method in order to check the recovery, using the modified extraction technique for LLM, *leucaena* seeds and excreta, as well as the analysis by HPLC.

Recovery of 3,4-DHP from LLM was accomplished by supplementing the LLM with crystalline 3,4-DHP at two levels prior to extraction. The initial concentration of 3,4-DHP in LLM was determined by comparing the respective detector responses for standards and the LLM extract. Recovery was calculated as the ratio of peak area obtained to the expected total peak area. Recovery of 3,4-DHP from excreta was determined by adding crystalline 3,4-DHP to freeze-dried

excreta, prior to extraction as described previously in this chapter. Chromatographic recovery was also determined by adding a standard solution to the extract prior to chromatography. This technique also gives an indication of the extent of interference of any closely eluting peaks.

Determination of the recovery of mimosine and 3,4-DHP in serum was estimated by the addition of $1cm^3$ of a standard solution (0.125mM mimosine; 0.25mM 3,4-DHP.) to $1cm^3$ of mimosine- and 3,4-DHP-free serum. Protein was precipitated by addition of $0.5cm^3$ of either SSA or PTA followed by treatment as described above. The effect of adding either of the precipitants to the standard solutions was assessed by adding $0.5cm^3$ of the precipitant to $2cm^3$ of the standard solution (0.125 and 0.25mM mimosine and 3,4-DHP respectively) prior to further treatment.

2.3.4 Chromatography.

An Altex liquid chromatography system was used for the chromatography. It consisted of an Altex Model 110A pump, a Rheodyne 7120 injection valve with a 20 ul loop, and an Altex-Hitachi Model 100-10 variable wavelength UV detector. The initial column choice was a 4.6 x 250mm Altex RP18 column packed with 10um C18 bonded silica giving an efficiency of 26,000 plates per meter (i.e. N=26,000) as measured using naphthalene as the analyte and a methanol:water mixture (70:30) as the eluent at a flow rate of $1cm^3\ min^{-1}$. During method development such purchased columns became unuseable due to high back pressures. The columns were repacked 'in house' with LiChrosorb RP18 (dp=10um) packing material and all further development work and subsequent analysis was conducted using these columns. The columns were packed using a Magnus P6050 column packer (Magnus Scientific, Cheshire, U.K.). Packing was achieved by slurrying the material in propan-1-ol (4.2g + $20cm^3$) and placing the slurry in an ultrasonic bath prior to packing. The column was packed upwards at 5000 psi, using degassed methanol H_2O (80:20) containing sodium acetate (0.2g dm^3) then prepared further as described elsewhere (Knox, 1982). The column was then conditioned by pumping with aqueous methanol (80:20 methanol:H_2O) at $1cm^3\ min^{-1}$. The column was tested using a test mixture of benzyl alcohol, toluene and naphthalene. The efficiency of the columns, measured using the naphthalene peak, were about 20,000 (N=20,000).

All eluents were prepared from 'in glass' double distilled water which had been collected in a glass container. It is considered imperative that eluents and their component solvents be stored in glass containers since it is possible for small amounts of plasticisers to leach from plastic containers into the eluent

components and be irreversibly bound to the column packing material. This may have caused the high back pressures in the original columns which could not be alleviated by the replacement of the top few mm of column packing material. All eluents were filtered through a Whatman GF/F glass fibre filter and degassed ultrasonically prior to use.

During the developmental period the chromatographic conditions (pH, buffer composition, organic modifier content and type, absence of, and presence of various types of ion pairing agent) were varied in an iterative fashion in order to obtain, or reduce retention and achieve resolution of mimosine and 3,4-DHP. The eluent which resulted had to be modified when extracts were chromatographed and further modified for serum samples. The eluent used for the isocratic analysis of mimosine and 3,4-DHP was prepared by mixing 200cm^3 of 0.01M sodium octyl sulphate (SOS) in 2% v/v aqueous methanol with 240 cm^3 of methanol. Sodium nitrate was added (5.1g) and the mixture made to 2dm^3 with distilled H_2O. The eluent was then brought to pH 2.25 with HNO_3 (ca. 7.9M) prior to filtering and degassing. Methanol was HPLC grade and purchased from Rathburn Chemicals Ltd, Walkerburn, Peeblesshire; SOS was obtained from Kodak, Liverpool while sodium nitrate was analytical grade (BDH Ltd, Poole, Dorset).

Chromatographic response to the analytes was ascertained by injection of standards of varying concentration and measuring the peak areas. Purity of the peaks was confirmed by calculating the peak area ratios. This involved running the standards and detecting these at 278 and 269nm and comparing the peak areas of samples treated in the same manner. Equivalent peak area ratios indicate minimal or no interference from other compounds.

2.3.4.1 HPLC using electrochemical detection (ECD).

The presence of phenolic OH groups on both the mimosine and 3,4-DHP nuclei make both compounds ideal candidates for detection using ECD rather than UV. Electrochemical detection has the advantage of being much more sensitive and specific than UV detection. The use of ECD was thus also subsequently investigated and provided another means of testing the purity of both mimosine and 3,4-DHP.

Isocratic chromatographic resolution of mimosine and 3,4-DHP was accomplished using a Biophase ODS (dp= 5um) column (4.6 x 250mm; Bioanalytical Systems Inc., West Lafayette, USA) at an eluent flow rate of 1cm^3 min^{-1}. The eluent used was based on that for the analysis of catecholamines

(Mayer and Shoup, 1983) and was prepared by dissolving monochloroacetic acid (28.3g), NaOH (9.4g), Na_2-EDTA and sodium octyl sulphate (0.45g) in about 500cm^3 double distilled H_2O as specified above. The solution was then made to 2dm^3 with distilled H_2O and adjusted to pH3 with 6M HCl. Acetonitrile (70cm^3) was added to the buffer and the resultant solution filtered and degassed as above. Tetrahydrofuran (THF; 36cm^3) was added prior to gentle mixing and placing in a sealed glass bottle. The necessity to filter, degas and place the eluent in a closed system is due to the volatility of THF thus making the THF content of the eluent difficult to control. Detection was by a Bioanalytical systems LC3A amperometric detector with the voltage set at +0.72V.

2.4 Analysis of canavanine and canaline.

The method proposed for the analysis of canavanine and canaline was to produce the OPA derivatives prior to separation using reversed phase HPLC with fluorescence detection.

2.4.1 Materials used.

Acetonitrile (S grade), methanol and THF were obtained from Rathburn Chemicals, Ltd. Canavanine, canaline, mercaptoethanol, OPA and amino acid standards were purchased from Sigma Chemicals, Ltd. Propanoic acid (which was distilled in this laboratory prior to use), OPA (Sepramar), dimethyl sulphoxide (DMSO) and other reagents were obtained from BDH Ltd. Spherisorb ODS2 (dp = 5um) was purchased from Phase Separations Ltd. (Queensferry, Clwyd) while columns and ODS-Hypersil (dp =3um) were obtained from Shandon Ltd (Runcorn, Cheshire).

The packing material was slurried in acetone and packed into the columns using degassed aceton- as the follower eluent. Other packing details are as described above, however the packer was modified to pack Shandon columns and use a Shandon packing tube. The column dimensions were 5 x 160 and 5 x 100 mm respectively for the 5 and 3um materials.

At a flow rate of 1cm^3 min^{-1} using an acetonitrile/H_2O (80:20) eluent and anthracene (in methanol) as the analyte the efficiency of the columns were high (N= 50,000-60,000 and 100,000-120,000 for the 5 and 3um columns respectively). Detection was by fluorescence using a Gilson model 121 fluorometer fitted with a 9ul flow-cell. E x citation and emission filter bandwidths were 305-395 and 420-650nm respectively. The gradient chromatograph system used for method development and analysis of canavanine and canaline was further composed of two Gilson model 302 pumps fitted with 5cm^3 heads. The gradient was controlled by an Apple II computer with Gilson software (gradient manager, 702, V1.2).

Injection of the sample was accomplished with a Rheodyne 7125 valve fitted with a 20ul loop.

2.4.2 Preparation of reagents.

The method is based upon that developed for amino acid analysis of physiological fluids using OPA as a precolumn derivatisation reagent (Turnell and Cooper, 1982). The preparation of the acetonitrile/ mercaptoethanol protein precipitant, the OPA reagent and the phosphate propionate buffer have been described elsewhere (Turnell and Cooper, 1982). Borate buffer (0.4M; pH 9.5) was prepared by dissolving boric acid in about $80cm^3$ water and adjusting to pH 9.5 with 4MNaOH prior to making to $100cm^3$. Standard mixtures of amino acids were purchased (Sigma Chemicals Ltd.) and diluted as appropriate with a solution of 9mM phenol in 0.1M HCl to yield a concentration of 0.25mM with respect to each amino acid. Further dilutions were made with distilled H_2O. Canavanine and canaline were purchased (Sigma Chemicals Ltd.) and the appropriate quantity of the crystalline material dissolved in distilled H_2O to give 0.25mM solutions. All amino acid standards were stored at -20°C until required for use. All eluents were filtered and degassed as above.

2.4.3 Preparation of samples.

Serum samples were obtained as described (2.12.3 & 2.3.2). Precipitation of protein was achieved by placing (by syringe) 40ul of serum into the bottom of a $2cm^3$ polypropylene centrifuge tube followed by 200ul of acetonitrile precipitant. After capping and mixing the tube was placed in a cooled (0-5°C) centrifuge and centrifuged at 12,500g for 2min. The resultant supernatant was decanted off for chromatography.

Jack beans and oven dried chick excreta were ground to pass a 1mm aperture and 0.5g weighed into a $250cm^3$ volumetric flask. After making to the mark with distilled H_2O the suspension was placed in an ultrasonic bath for 20min. An aliquot of the suspension was then passed through a 0.5um millex membrane filter (Millipore Ltd, Harrow, U.K.). The colourless filtrate was taken for chromatography immediately or stored at -20°C until required for use. Prior to chromatography the filtrate was treated with acetonitrile precipitant as described above. In order to confirm the efficiency of the extraction procedure and also to show the absence of any contaminant peaks, samples of JB and soya bean were hydrolysed and the hydrolysate taken for chromatography. The procedure for the acid hydrolysis of JB and soya bean has already been described (D'Mello, 1973).

Table 2.4.1 Composition of the eluents used for the chromatographic determination of canavanine and canaline.

eluent	A	B	H
Component (cm^3)			
H_2O	720	420	590
tetrahydrofuran	---	---	380
acetonitrile	80	300	---
methanol	---	250	---
dimethyl sulphoxide	---	30	30
propionate buffer	300	---	---

Eluent D is composed of A (750 cm^3) and B (250 cm^3)

Table 2.4.2 Gradient conditions used for the resolution of canavanine and canaline with Spherisorb ODS and ODS Hypersil columns.

a. Spherisorb ODS.

time (min)	proportion of eluent D	H
0.0	1.00	0.00
8.0	1.00	0.00
8.1	0.88	0.12
36.2	0.88	0.12
36.3	0.30	0.70
40.0	0.30	0.70
40.2	0.00	1.00
44.0	0.00	1.00
44.2	1.00	0.00
50.0	1.00	0.00

b. ODS Hypersil

time (min)	proportion of eluent D	B
0.0	1.00	0.00
5.5	1.00	0.00
6.0	0.90	0.10
7.0	0.00	1.00
11.0	0.00	1.00
12.0	1.00	0.00
15.0	1.00	0.00

2.4.4 Recovery of canavanine and canaline from samples.

Recovery of canavanine and canaline from serum was accomplished by the addition of 20ul of the appropriate standard to 20ul of serum, prior to precipitation of the protein. Recovery from JB and excreta necessitated the addition of both amino acids to samples of both jack beans and excreta prior to extraction. The canavanine was added as the crystalline solid whereas canaline was added in the form of a solution in water because of the small quantities available and high cost. The extraction procedure was reduced in scale by two orders of magnitude so that 50mg of sample was weighed and made up to 2.5 cm^3.

2.4.5 Method of derivatisation and chromatography.

Aliquots (20ul or 50ul for some serum samples) of the protein free serum samples were added to 200ul (170ul when 50ul of sample was taken) of OPA reagent in a $2cm^3$ polypropylene centrifuge tube and mixed immediately. A 50ul sample was taken by syringe and injected onto the column 25s after addition of the sample to the OPA.

The chromatographic conditions were based on those used for the analysis of amino acids in serum and urine (Turnell and Cooper, 1982). From the data presented by these workers and from the structurally and physically similar characteristics of canavanine and arginine it was expected that retention times of arginine and canavanine would be similar. Initial development work was carried out using the 5 x 160mm column packed with Spherisorb ODS2 (dp=5um). The buffered eluents used are shown in Table 2.4.1. Eluent H was developed using the Simplex procedures referred to in chapter 1 (sect. 1.13). It was necessary to adopt this method because of the considerable difficulty encountered in resolving canaline from the other amino acids. The gradient conditions used for Spherisorb ODS and ODS Hypersil are presented in table 2.4.2 a and b respectively. Another gradient was also developed for the analysis of canavanine with the Spherisorb ODS column within 15 min.

The use of the novel packing material, pyrolised graphitic carbon for the analysis of conventional amino acids and canavanine was tested. The material packed in a Shandon column (5 x 160mm) (supplied by B. Kaur, Wolfson Liquid Chromatography Unit, Edinburgh University) showed completely different selectivities with the standard amino acid mixture compared to the ODS packing material; good peak shapes being obtained. After some time of use, however, peak shape and reproducible retention times were lost and were not recoverable.

This may have been caused by irreversible adsorption of artifacts onto the packing material. Further development work for the analysis of canavanine and canaline using this column ceased but such a packing material may provide a method for the rapid analysis of compounds such as canaline which are difficult to resolve by conventional reversed phase HPLC.

2.4.6 Quantitation.

Standard curves were prepared by treating 40ul aliquots of the appropriate standard with precipitant followed by treatment as described above. The relationship between peak area and concentration of standard was plotted. The peak areas of canavanine and canaline from samples were calculated and concentration of these obtained by reference to the respective peak areas of the external standard. During the method development stage retention times and peak areas were measured by hand. Latterly these were obtained from a Trio chromatography computing integrator (Trivector Systems Int. Ltd, Bedfordshire, U.K.).

2.4.7 Determination of canavanine using colorimetry and ion exchange chromatography.

Analysis using IEC was achieved using the normal running conditions with a Technicon AA1 amino acid analyser (D'Mello, 1973). Chromatography of the acid hydrolysate yielded the canavanine peak, with a retention time of about 4.9h, just prior to that of arginine. Detection was accomplished by post column reaction with TNBS (D'Mello, 1973, Acamovic and D'Mello, 1981) with colorimetric detection. Quantification was achieved by relating the peak area to that for the internal standard, nor-leucine.

Colorometric analysis of canavanine in JB was achieved using a modification of the PCAF method (Rosenthal, 1977). Extraction of canavanine was as described above (sect. 2.4.3). The filtrate ($5cm^3$) was mixed with phosphate buffer ($5cm^3$), potassium persulphate solution ($1cm^3$) and activated PCAF solution ($0.5cm^3$), mixing after each addition, and allowed to stand for 40min. The absorbances were obtained at 530nm and compared with those from standard canavanine solutions (0-1.0mM).

2.5 Large scale extraction and purification of canavanine from jack beans.

In order to assess the effects of canavanine on the growth of poultry without the results being confounded by the presence of other potentially deleterious

components, supplementation of a normal diet with canavanine was considered necessary. Supplementation of diets with substantial quantities of canavanine is prohibitive in terms of cost thus necessitating the isolation and purification of canavanine from JB. The method of extraction and purification involved the combination and modification of various techniques used in the isolation of amino acids from plant material (Hirs, Moore and Stein, 1954; Hunter, Houston and Kester, 1955; Hegarty and Court, 1964; Natelson and Bratton, 1984).

2.5.1 Method of extraction.

Jack beans (3kg; canavanine content = 26g kg^{-1}) were ground to pass a 1mm aperture. The resultant flour was weighed (100g) into ten $1dm^3$ polypropylene centrifuge bottles and about $900cm^3$ of distilled water added. A (25-30cm length) dialysis tube (Visking 20/32) filled (15-16cm bed height) with a slurry of ion exchange resin (Dowex 50W-X8(H); 16-40 mesh) in gum arabic solution (7.5g acacia powder dm^{-3} deionised H_2O) was placed in each bottle and the cap screwed on. The bottles were then placed on an end over end shaker (rotation rate : about 44 revolutions min^{-1}) for 24h. The dialysis tubes were replaced and the bottles rotated for a further 48h at which time the tubes were removed and the resin pooled.

Prior to slurrying, the resin was pretreated by washing with deionised water (about 4 bed volumes) then with ethanol (GPR; 4 bed volumes), deionised H_2O (2 bed volumes), 2M NaOH (2 bed volumes), H_2O (4 bed volumes), 2M HCl (2 bed volumes) then H_2O until free from acid. The ethanol wash removed a considerable quantity of orange material from the new resin. The preparation of the resin was carried out using a Buchner flask and filter. The resin was allowed to stand in each volume of each of the washing liquids for two or three minutes, with occasional gentle stirring using a glass rod, prior to suction being applied. When pretreatment of the resin was complete it was suspended in the gum arabic solution prior to slurrying into the dialysis tubing.

The pooled resin containing the extracted canavanine was washed in a Buchner funnel with deionised H_2O (10 x $200cm^3$) and ethanol (3 x $200cm^3$) followed by deionised H_2O (3 x $200cm^3$). At each stage the filtrate was collected and tested for the presence of canavanine by HPLC. Canavanine was then eluted from the resin using NH_4OH solution ($100cm^3$ volumes of 4M) until canavanine ceased to be eluted (about $1800cm^3$). Elution of canavanine using 0.16M NH_4OH or 2M NH_4OH, which had been used for the preparation of canavanine and mimosine respectively, (Hegarty and Court, 1964; Natelson and Bratton, 1984) was unsuccessful in eluting canavanine from the resin. The lack of success with 2M

NH_4OH was presumably due to the increased basicity of canavanine compared to mimosine while the use of 0.16M may have been unsuccessful due to the resin being in the H form rather than the NH_4 form. The presence of canavanine was detected by sampling the filtrate, evaporating off the ammonia which severely interferes with the derivatisation procedure, and redisolving the residue in H_2O prior to chromatography.

The eluate from the resin was evaporated to about half the volume in a thin film rotary evaporator and then lyophylised. The lyophylisate was washed with small portions of ethanol and again lyophilysed. This procedure realised 39g of canavanine of 95% purity as measured using HPLC. This return of canavanine from 3kg of JB seed is very low (50% of possible yield) compared with that reported by other workers (Natelson and Bratton, 1984) although their method is more time consuming and precise.

The resin remaining after extraction of canavanine was washed with deionised H_2O prior to treatment as described above and then used for the extraction of more canavanine.

2.6 Qualitative examination of carbohydrates in lupins.

Treatment of lupin seeds with enzymes was hoped to increase the proportion of lower molecular weight carbohydrates thus making the treated seed more acceptable as an ingredient in poultry diets. HPLC was used to qualitatively assess the degree of enzyme attack and to give an indication of the type of carbohydrate produced.

2.6.1 Sample preparation for analysis by HPLC.

Samples (1g) of untreated or enzyme treated lupin seed were suspended in $20cm^3$ of distilled H_2O in dialysis tubing (Visking, 20/32) and dialysed overnight at $4^{\circ}C$ against $250cm^3$ of distilled H_20. The resultant dialysate was then passed through a column (10 x 160mm) of mixed bed ion exchange resin (Amberlite monobed MB1; analytical grade, BDH Ltd.) under gravity. The first $10cm^3$ of eluent were discarded and about the next $30cm^3$ retained. Twenty five cm^3 of the eluent were dried down under rotary evaporation.

2.6.2 Chromatography.

Chromatography was conducted using a Gilson HPLC system using two columns (HP-87P and Neucleosil-5-NH2). The details and conditions used have been

described in the literature (Sutherland and Kennedy, 1986; Kennedy and Sutherland, 1986; in press).

2.7 Qualitative examination of saponins in lupin seed, soya beans, *leucaena* leaf meal, *leucaena* seed and jack beans using thin layer chromatography.

A qualitative and semi-quantitative assessment of the saponin content of the above named legumes was undertaken using TLC. Soya beans were used for comparative purposes since the technique used here has been shown to be effective in detecting saponins in these (Curl, Price and Fenwick, 1985).

2.7.1 Sample preparation.

The method of sample preparation was based on that described by other workers (Curl, Price and Fenwick, 1985). The finely ground material (to pass a 1mm aperture) was weighed (5.1g) into an extraction thimble and extracted (8h) with petroleum spirit (bp. 40-60°C) using a soxhlet extraction unit. After the residue remaining in the thimble was dry the saponins were similarly extracted with methanol for 24h. The extract was evaporated to dryness using a thin film rotary evaporator and 5cm^3 of distilled H_2O added. The flask was warmed (40°C) to assist dissolution of the residue however all the residue did not dissolve. The supernatant solution (0.5cm^3) was passed through a Sep-Pak cartridge (C18) and the cartridge washed with 20cm^3 of distilled H_2O. The Sep-Pak was then washed with 10cm^3 of methanol to elute the saponins. The resultant solution was evaporated to dryness and 0.5cm^3 of methanol added.

2.7.2 Chromatography.

The methanolic solution of saponins (10ul) from each of the samples were spotted onto a precoated silica gel plate (layer thickness = 0.2mm ; E. Merck, Darmstadt, F.R.G.). Saponin white (BDH Ltd, U.K.) standard solutions in distilled H_2O (0.05-5.0 mg cm-3) were also spotted alongside the extracts. The chromatogram was developed using a butan-1-ol : ethanol : ammonia solution (7:2:5) as described elsewhere (Curl, Price and Fenwick, 1985). The developed plate was dried and sprayed with a mixture of anisaldehyde (3-methoxybenzaldehyde): glacial acetic acid :concentrated sulphuric acid (1:100:2) and dried in an oven at 120°C for 15min. The Rf values of each spot were determined and the intensity of the colours from the extracts related to those of the standards, visually. All the samples used, produced spots with the same Rf (0.075) as saponin white. The intensity of the colours were within the range of

Table 2.8.1 Trypsin inhibitor activity (decrease in 3-nitroanaline conc./ 3-nitroanaline conc. in uninhibited solution) of Leucaena leaf meal (LLM), Leucaena seeds, jack beans, lupins and winged beans.

sample No.		trypsin inhibition (mean of two analysis)
1.	LLM (sun-dried, unpelleted) cv "Peru" ex Malawi 1977	0.081
2.	LLM (sun-dried, pelleted) cv "Peru" ex Malawi 1979	0.223
3.	as 2 + autoclaved wet at 121 C for 30 min*	0.163
4.	as 3 + PEG4000 + Fe(III)*	0.000
5.	as 2 + incubation with enzymes£	0.000
7.	Leucaena seeds ex Mexico 1981	0.817
8.	jack beans ex Mexico	0.068
10.	jack beans autoclaved at 121°C for 1h+	0.060
11.	lupin seeds; *Lupinus albus*, cv Vladimir [Kievskji mutant]	0.000
12.	winged bean	0.734
13.	winged bean, autoclaved at 121°C for 30 min	0.000

* See text of "diets" in section 2.13.5.
£ See text of "diets" in section 2.13.6.
+ See text of section 2.13.10.

standards. This indicated that saponins were present in all samples tested and the concentrations were in the range 2.2-11.3 g kg^{-1} DM.

2.8 Trypsin inhibitor assay.

Trypsin inhibitor was assayed using the synthetic substrate, RS benzoyl-arginine-3-nitroanalide (BANA; Erlanger, Kokowski and Cohen, 1961). Under the action of trypsin, BANA is hydrolysed to yield 3-nitroanaline, causing a bathochromic shift and thus allowing the amount of 3-nitroanaline to be measured spectrophotometrically at 410nm. The presence of inhibitors hinders the formation of 3-nitroanaline.

2.8.1 Sample preparation and assay procedure

Samples were prepared using a modification of the method described previously by other workers (Balogun and Koch, 1979). The sample (2g, ground to pass a 1mm aperture) was extracted in a soxhlet thimble with petroleum spirit (bp. 40-60 °C) for 6h prior to extraction of the defatted meal with Tris buffer. The extraction was achieved by placing the sample and Tris buffer, in a centrifuge tube in an ultrasonic bath for 5min. prior to centrifugation for 10min at about 2000g. The trypsin solution used contained 500mg trypsin (EC 3.4.21.4; from porcine pancreas, typeII; Sigma chemicals, Poole, U.K.) in 1 dm^3 of 0.001M HCl. Trypsin inhibitor activity was determined relative to the trypsin and BANA without the presence of the Tris extract according to the method previously described (Balogun and Koch, 1979).

2.8.2 Trypsin inhibitor activity of samples tested.

The inhibitor activity is expressed (Table 2.8.1) as the degree of inhibition of release of 3-nitroanaline in the presence of the test extract, relative to its release by uninhibited trypsin. The samples used for trypsin inhibitor assays were LLM, *leucaena* seeds, lupin seeds jack beans and, for comparison, winged beans. The quantity of sample taken for extract was 0.1g in all cases.

From table 2.8.1 it can be seen that the trypsin inhibitor activity of LS is as potent as that of unheated winged beans. Autoclaving winged bean effectively destroys the trypsin inhibitors present but it has little effect on the low activity trypsin inhibitors in jack beans. Heating, however, slightly reduced the inhibitor activity present in LLM. Preincubation of LLM with enzymes and supplementation with PEG4000 and Fe(III) prior to autoclaving, removed the trypsin inhibitor activity in LLM. The seeds of lupin showed no trypsin inhibitor

activity. A solution of mimosine, containing the equivalent concentrations as those in the LLM meal obtained from Malawi in 1979, slightly inhibited (0.046) trypsin activity.

2.9 Urea and ammonia assay.

Two enzymatic methods were used for the determination of ammonia and urea. Both were purchased as kits for the determination of urea (Boehringer Corporation (London) Ltd, U.K.) and the test modified slightly to enable NH_3 to be determined. The kit which utilised the formation of the blue indophenol from ammonia, hypochlorite and alkaline phenate (Berthelot reaction) was not sensitive enough to detect ammonia present in the serum samples. It was also considered insufficiently specific (Ngo, Phan, Yam and Lenhoff, 1982) to accurately estimate any free ammonia in the serum.

The other more specific and sensitive method involved monitoring the decrease in NADH concentration in a coupled enzyme reaction at a wavelength of 340nm. In this method the background NH_4^+ can be determined prior to the addition of urease. The determination of NH_4^+, produced by the degradation of urea, can therefore be similarly measured and thus the concentration of urea calculated.

2.10 Method used for the UV measurement of ammonia and urea.

Serum samples (100ul) were added to 2.5cm^3 of buffer containing NADH, GLDH and a-oxoglutarate. The solution was mixed and incubated at room temperature for 10min. prior to measurement of the absorbance at 340nm in a spectrophotometer (SP8-100; Pye Unicam, Cambridge, U.K.) against a reagent blank. The urease suspension (10ul) was then added, the cuvettes mixed and allowed to remain at room temperature for a further 15min. before measuring the absorbance. This change in absorbance was due to the ammonia released from any urea present in the sample.

2.11 Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assays in serum.

The methods used for the measurement of serum ALT and AST activities are similar. Both assays depend on monitoring the reduction in NADH concentration during the reaction. The reduction in NADH was measured spectrophotometrically at 340nm using the procedure described in the kits (Boehringer Corporation (London) Ltd, U.K.).

Analysis of serum for enzyme activity and for other components were performed on the serum samples taken from individual birds.

2.12 General conduct of chick experiments.

2.12.1 Experimental design.

Animal experiments were arranged in a randomised block fashion except for experiment 4 which was conducted as a latin square.

2.12.2 Statistical analysis of results.

Data obtained were statistically analysed by analysis of variance (ANOVAR) of a randomised block design and differences tested for significance using Students-t test. Data in experiment 4 were analysed using ANOVAR of a latin square design and tested for significance by Students-t test. Mimosine and 3,4-DHP output/intake ratios were compared for differences by obtaining the standard error of the mean (sem) of each for each treatment and tested using Students-t test (P. Phillips, personal communication).

2.12.3 Allocation of chicks, preparation of diets and ingredients and collection of samples

Male broiler chicks (Marshall LB9 strain vaccinated against infectious bronchitis) were obtained at one day of age from a local hatchery (D.B. Marshall, Whitburn, West Lothian) and housed, 7 or 8 to a cage, until they were seven days of age (day 7). The cubic cages ($38cm^3$) were constructed from stainless steel wire. The excreta was collected in trays below each cage. Food and water were available, ad libitum, from stainless steel troughs fixed to the front of the cages. The composition of the starter diet fed to the chicks during this period is presented in Appendix 1.

The cages were arranged in four layers (replicates) within a thermostatically controlled room. Light was provided for 22h each day and the temperature regime was as follows; day 1: 34°C, day 7: 29°C, day 14: 27°C and day 21: 24°C; where day 1 is the first day the birds are caged.

When the birds were seven days of age they were wing tagged and weighed, the tag numbers and corresponding bird weights being entered into a Commodore CBM micro computer (model 3032). The computer allocated the birds to cages according to their body weights; the lightest being allocated to the top replicate and the heaviest on the lowest replicate within an experiment. Although replicate layers were constrained to a weight range, birds within a replicate were allocated

randomly. In experiment 1, chicks were allocated manually to the cages minimising weight differences within each replicate as described above.

Birds were allocated so as to minimise the weight range within a replicate thus reducing variation caused by having large and small birds in the same cage. Chicks were also individually weighed at day 14 and day 21 unless otherwise stated. In experiments 1 to 7 food was weighed into the food troughs and at the end of the experiment the residue remaining in the troughs was weighed. In subsequent experiments a more rapid technique was used. Food was added to the troughs from preweighed and labelled polypropylene containers containing sufficient diet for each cage. The total diet remaining at the conclusion of experiments was weighed, the difference between start and finish was the diet consumed per cage.

When necessary blood was drawn by syringe from the heart. Where carcass analysis was conducted chicks were killed by cervical dislocation and the dead birds placed in storage (-20°C) until they were able to be processed. Processing of the carcasses was by the method previously described (D'Mello and Acamovic, 1976).

Excreta was collected in dilute sulphuric acid on polythene covered trays, transferred to sealed containers and stored at -20°C for experiments 1 to 3. When required for analysis the contents of the container were homogenised and aliquots taken for dry matter determination and for extraction. In subsequent experiments where excreta was collected, all the excreta collected during the collection period was transferred to pre-labelled and weighed aluminium dishes which were then placed in an oven at 100°C for three days, for drying. The dried material, in both cases, was ground in a mill to pass a 1mm aperture prior to analysis. Diets, and ingredients of diets were similarly ground prior to analysis.

All diets were mixed in a Hobart mixer prior to allocation to chicks. The starter diet was based on maize, soya meal and fishmeal with supplemental minerals and vitamins. The composition of the starter diet, mineral and vitamin supplements are presented in appendix 1. All diets were designed to have a sufficient supply of essential nutrients as recommended by the Agricultural and Food Research Council (ARC, 1975). Ensured adequacy was achieved by the addition of minerals, vitamins and methionine (Appendix 1)

When preparing the mineral supplement the dicalcium phosphate and limestone flours were weighed into the container first and then the other ingredients added one at a time mixing well after each addition. This procedure prevents reaction

Table 2.13.1 Composition of diets used in experiment 1.

ingredients (g kg^{-1})	diet No. 1.1	1.2	1.3	1.4	1.5	1.6	1.7
maize meal	428.7	408.7	388.7	416.7	404.7	396.7	364.7
soya bean meal	310.0	310.0	310.0	310.0	310.0	310.0	310.0
minerals & vitamins	43.3	43.3	43.3	43.3	43.3	43.3	43.3
Leucaena leaf meal~	150.0	150.0	150.0	150.0	150.0	150.0	150.0
vegetable oil	65.0	65.0	65.0	65.0	65.0	65.0	65.0
(RS)methionine	3.0	3.0	3.0	3.0	3.0	3.0	3.0
PEG4000*	---	20.0	40.0	---	---	20.0	40.0
Fe2(SO4)3^	---	---	---	12.0	24.0	12.0	24.0
dietary components (by analysis, in DM)							
ash	67.8	68.0	67.7	76.3	80.7	74.1	79.8
nitrogen	39.5	40.4	38.7	39.0	36.8	37.6	38.3
fibre (TCA)	98.0	100.0	105.0	102.0	102.0	103.0	108.0
gross energy ($MJkg^{-1}$)	19.7	19.5	20.2	19.5	19.1	19.7	19.4
iron ($mgkg^{-1}$)	228	224	192	2540	4816	2453	4143
pH@	5.8	5.9	5.9	5.4	5.0	5.3	5.0
pH	5.7	5.8	5.8	4.8	4.4	4.8	4.3

* polyethylene glycol (RAM=4000; Sigma Chemicals, Poole, U.K.)
^ ferric sulphate (GPR; BDH, Poole, U.K.); Fe content determined to be 198.9 g kg^{-1} using atomic absorption spectrometry.
~ Peru cultivar.
@ macerated (see text)
shaken (see text)

Table 2.13.2 Composition of leaf meal from Leucaena leucocephala leaf meal (cv. Peru) used in experiment 1.

component	(g kg^{-1} dry matter)
ash	70.0
nitrogen	46.6
trichloroacetic acid-fibre	89.1
ether extract	47.7
gross energy ($MJkg^{-1}$)	19.6
tannin*	33.6
mimosine (determined by HPLC)	25.5
3,4-DHP (determined by HPLC)	5.1
saponin (estimated by TLC)	2.2-11.3

* determined using the AOAC (1970) method.

occurring between the ingredients and is particularly important for addition of ingredients with oxidising and reducing properties. The final mixture was blended thoroughly in a Hobart mixer.

2.13 Chick experiments.

A brief summary sheet of diets fed during the course of this work may be found inside the rear cover of this work.

2.13.1 Experiment 1.

Previous work (D'Mello and Acamovic, 1982) has shown that excretion of mimosine from chicks fed LLM diets was higher when diets were supplemented with Al(III) rather than Fe(II). Also inclusion of polyethylene glycol into diets containing sorghum has been shown to be effective in reducing the adverse effects caused by the presence of tannins (Ford and Hewitt, 1979b; 1979c).

Object

This experiment was conducted to investigate the effect of supplementing LLM based diets with Fe(III) and polyethylene glycol (RAM =4000: PEG4000), separately and in combination.

Diets

A brief summary of the diet codes for all experiments are available in pullout sheets inside the rear cover of this work.

The diets were designed to be adequate in all essential nutrients and details of their composition are presented below (Table 2.13.1). The diets were calculated to have crude protein and AME values of 217 g kg^{-1} and 12 MJ kg^{-1} respectively.The inclusion levels of ingredients for all diets are given as g air dry ingredient in each kg of air dry diet. The composition of the diets with respect to tannin, mimosine and 3,4-DHP, from the supplementary LLM, were calculated to be 4.8, 3.5 and 0.7 g kg^{-1} of diet dry matter. Ferric sulphate was added at levels such that the molar ratios of Fe(III) to mimosine would be about 2.5:1 and 5.0:1. These ratios were chosen because earlier work (D'Mello and Acamovic 1982) had shown that Al(III) added to LLM diets, elicited the greatest excretion of mimosine ingested, at a ratio of 5.4:1. Ferrous sulphate supplemented diets reduced excretion of mimosine but the ratio of Fe(II): mimosine was 2.6:1.

Table 2.13.3 Composition of diets used in experiment 2.

ingredients (g kg^{-1})	diet No. 2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8
maize meal	580.0	575.2	568.0	418.7	417.1	397.1	413.9	393.9
soya bean meal	374.0	374.0	374.0	320.0	320.0	320.0	320.0	320.0
Leucaena ~ leaf meal	---	---	---	150.0	150.0	150.0	150.0	150.0
minerals & vitamins	43.3	43.3	43.3	43.3	43.3	43.3	43.3	43.3
vegetable oil (RS)	---	---	---	65.0	65.0	65.0	65.0	65.0
methionine	2.7	2.7	2.7	3.0	3.0	3.0	3.0	3.0
PEG4000*	---	---	---	---	---	20.0	---	20.0
Fe2(SO4)3^	---	4.8	12.0	---	1.6	1.6	4.8	4.8
dietary components (by analysis,in DM)								
ash	69.1	74.1	76.6	72.6	72.8	73.9	75.9	75.8
nitrogen	39.4	35.5	33.4	39.2	37.9	39.8	36.9	37.3
fibre (TCA)	43.0	58.0	60.0	107.0	96.0	95.0	104.0	97.0
gross energy ($MJkg^{-1}$)	17.9	17.7	17.5	19.6	19.4	19.6	19.4	19.7
iron (mg kg^{-1})	200	1059	2589	221	528	538	1136	1126
pH@	6.2	5.9	5.5	5.9	5.8	5.8	5.5	5.6
pH	6.1	5.5	5.1	5.8	5.8	5.8	5.4	5.4

~ Peru cultivar prepared as in experiment 1
* polyethylene glycol (RAM 4000; Sigma Chemicals Ltd., Poole, U.K.)
^ ferric sulphate (GPR; BDH, Poole, U.K.); Fe content determined to be 198.9 g kg^{-1} using atomic absorption spectrometry.
@ macerated (see text of experiment 1).
shaken (see text of experiment 1).

The *leucaena* (cv. Peru) used in this experiment was harvested in Malawi, sun-dried and pelleted. The pellets were ground prior to inclusion into the diets. The composition of the LLM used is presented in table 2.13.2.

The pH of each of the above diets were measured by two techniques. Maceration was carried out by homogenising 3g of diet in $100cm^3$ of distilled H_2O for about 30s. Homogenisation was done at the highest speed setting using an Osterizer (Oster Corp., Millwaukee, USA. Suppl. A. Gallenkamp, U.K.). The pH of the resultant suspension was measured. The other technique involved shaking 3g of the diet with $100cm^3$ of distilled H_2O for about 30s prior to measuring the pH of the mixture. The pH of the solutions containing 0.072 and 0.144g ferric sulphate in $100cm^3$ of distilled H_2O were 3.3 and 3.0 respectively.

2.13.2 Experiment 2.

Object

This experiment was designed to assess the effects of ferric sulphate on chicks fed control soya bean/maize diets and also to ascertain the effects of reducing the Fe(III):mimosine ratio in LLM supplemented diets.

Diets

The diets were prepared as described previously and included LLM, PEG and ferric sulphate from the same sources and prepared in the same manner therefore additional tannins, saponins, mimosine and 3,4-DHP from LLM are identical to those levels in experiment 1. Table 2.13.3 shows the composition of the diets. The diets were composed to contain about 218 g kg^{-1} and 12 MJ kg^{-1} of crude protein and AME respectively.

2.13.3 Experiment 3.

Object

The purpose of this experiment was to study the effect of feeding *leucaena* seeds at a level such that the mimosine content in the diet, from the seeds, was equivalent to that supplied by LLM. The effectiveness of Fe(III) on reducing mimosine toxicity from seeds could be observed and compared with that for LLM. A comparative assessment of the toxicities of LLM and the seeds would then be made.

Diets

The diets were designed to be adequate in all nutrients and were prepared as described in previous sections. Details of their composition, and that of the

Table 2.13.4 Composition of diets used in experiment 3.

ingredients g kg^{-1})	diet No. 3.1	3.2	3.3	3.4	3.5	Leucaena seeds
maize meal	573.3	416.2	506.9	503.4	476.2	---
soya bean meal	379.0	322.3	353.0	353.7	341.0	---
minerals & vitamins	44.9	44.9	44.9	44.9	44.9	---
vegetable oil	---	63.5	27.1	28.3	39.7	---
Leucaena leaf meal	---	150.0	---	---	---	---
Leucaena seeds	---	---	65.0	65.0	95.0	---
(RS) methionine	2.8	3.1	3.1	3.1	3.2	---
$Fe_2(SO_4)_3$	---	---	---	1.6	---	---
tannin[£] (g kg^{-1} DM)	---	0.39	0.46	0.46	0.67	7.1
mimosine (g kg^{-1} DM)	0.0	3.5	3.6	3.6	5.3	55.6
3,4-DHP (g kg^{-1} DM)	0.0	0.9	0.0	0.0	0.0	0.0
dietary components (by analysis, in DM)						
ash	70.7	76.3	72.7	75.2	70.4	38.4
ether extract	15.6	84.0	40.4	38.4	54.0	74.8
nitrogen	37.5	38.8	37.8	37.3	37.2	45.0
fibre (TCA)	57.6	48.4	56.8	56.8	59.6	123.2
gross energy (MJ kg^{-1})	17.8	19.5	18.5	18.5	18.7	20.2
iron (mg kg^{-1})	202	146	121	441	132	---

[£] tannin supplied from either Leucaena leaf meal or from Leucaena seeds and was determined in the original material using the AOAC (1970) method. mimosine and 3,4-DHP levels in the diets are calculated from their levels measured in the plant material using HPLC.

Table 2.13.5 Composition of diets in experiment 4.

ingredients (g kg^{-1})	diet No. 4.1	4.2	4.3	4.4
maize meal	570.4	411.5	408.0	384.6
soyabean meal	381.3	326.0	326.8	332.2
minerals & vitamins	44.9	44.9	44.9	44.9
Leucaena leaf meal	---	150.0	150.0	150.0
vegetable oil	0.6	64.6	65.8	73.4
(RS) methionine	2.8	3.0	3.0	3.0
$FeSO_4$*	---	---	1.55	11.9
dietary components (by analysis in DM).				
ash	70.4	84.8	80.5	74.2
ether extract	16.4	86.0	87.2	92.0
nitrogen	36.8	37.3	37.9	38.6
fibre (TCA)	52.8	52.0	53.6	44.0
gross energy (MJ kg^{-1})	18.0	19.5	19.8	19.6

* ferrous sulphate ($FeSO_4.7H_2O$, GPR; BDH, Poole, U.K); Fe content= 200.9g kg^{-1} as measured using atomic absorption spectrometry.

Table 2.13.6 Composition of diets used in Leucaena experiment 5.

ingredient (g kg^{-1})	diet No. 5.1	5.2	5.3	5.4	5.5	*5.6	*5.7	5.8
maize meal	528.6	507.3	380.5	309.3	330.6	380.5	309.3	521.1
soyabean meal	415.6	419.7	351.4	365.3	361.1	351.4	365.3	417.1
vegetable oil	8.1	15.2	70.1	94.0	86.9	70.1	94.0	10.6
minerals & vitamins	44.9	44.9	44.9	44.9	44.9	44.9	44.9	44.9
Leucaena leaf meal	---	---	150.0	150.0	150.0	150.0	150.0	---
(RS)-methionine	2.8	2.8	3.0	3.0	3.0	3.0	3.0	3.0
PEG4000	---	---	---	20.0	---	---	20.0	---
$Fe_2(SO_4)_3$	---	---	---	8.5	8.5	---	8.5	---
PVP-40	---	10.0	---	---	10.0	---	---	---
cholesterol	---	---	---	5.0	5.0	---	5.0	---
added mimosine	---	---	---	---	---	---	---	3.3

* Leucaena used in these diets was heat treated (see text) prior to incorporation into the diet.

The analysis of the diets used in experiment 5 are presented in table 2.13.7.

Table 2.13.7 Analytical composition of the diets used in Leucaena experiment 5.

composition (g kg^{-1} DM)	diet No. 5.1	5.2	5.3	5.4	5.5	5.6	5.7	5.8
ash	72.5	73.8	73.8	79.2	78.5	73.6	78.6	71.2
ether extract	32.8	38.8	99.2	124.4	115.2	94.0	125.6	34.8
nitrogen	39.7	40.6	38.9	38.2	38.2	41.5	40.6	40.3
gross energy (MJ kg^{-1})	18.3	18.7	19.9	20.8	20.0	20.3	20.6	18.6

leucaena seeds used, are shown in table 2.13.4. The saponin content of the seeds was determined to be in the range of about 2-11g kg^{-1} estimated visually from the TLC plates. The diets were calculated to be isonitrogenous and isoenergetic having crude protein and AME values of 217.5 g kg^{-1} and 11.8 MJ kg^{-1} air dry diet respectively. The supplemental $Fe_2(SO_4)_3$ was identical to that used in experiment 2.

2.13.4 Experiment 4.

Object.

The objectives of this experiment were to investigate the effectiveness of Fe(II) in the detoxification of LLM when Fe(II) was included at the same ratio to mimosine as Fe(III) in experiments 1 and 2. The experiment conformed to a latin square design.

Diets.

The diets were composed so as to supply 218 g of crude protein and 12 MJ of energy in each kg of air dry diet. The composition of each diet is shown in table 2.13.5 and were calculated to be adequate in all essential nutrients.

2.13.5 Experiment 5.

Object

The objectives in this experiment were to investigate the effects of Fe(III), polyethylene glycol (with a RAM of 4000; PEG 4000), polyvinylpyrrolidone (with a RAM of 40,000; PVP 40), heat treatment and cholesterol in diets containing LLM (150 g kg^{-1}). Cholesterol was added in an attempt to reduce any antinutritive effects caused by the presence of saponins while it was hoped that PEG and PVP would interact with any tannins thus reducing the antinutritive effects of these. A comparison of the effectiveness of PEG and PVP in reducing the adverse nutritional characteristics of LLM could then be made.

Diets.

The diets were calculated and prepared as described in previous experiments, to provide 217.5g kg^{-1} and 11.8 MJ kg^{-1} of crude protein and AME on an air dry basis.

The LLM used in diets 5.6 and 5.7 (Table 2.13.6) was treated prior to incorporation into the diets. The LLM used in diet 5.6 was placed in an autoclavable bag and distilled water added until the LLM was a wet dough. The mixture in the bag was placed in an autoclave at 121 °C for 30min prior to

Table 2.13.8 Composition of diets in Leucaena experiment 6.

ingredients (g kg^{-1})	diet No. 6.1	6.2	6.3	LLM
glucose	457.0	57.0	57.0	---
soyabean meal	70.0	70.0	70.0	---
maize meal	178.8	186.8	178.8	---
white fish meal	100.0	100.0	100.0	---
casein	100.0	100.0	100.0	---
vegetable oil	30.0	30.0	30.0	---
mineral & vitamins	44.9	44.9	44.9	---
$Fe_2(SO_4)_3$	8.0	8.0	8.0	---
Leucaena leaf meal	---	400.0	400.0	---
hemicellulase	11.3	3.3	11.3	---
composition (by analysis; g kg^{-1} DM)				
ash	69.3	106.1	110.0	107.8
nitrogen	31.8	48.2	47.7	47.1
gross energy (MJ kg^{-1})	17.6	19.4	19.2	19.7
fibre (TCA)	13.6	63.2	54.0	80.8
tannin	---	8.4	8.4	21.7
mimosine	---	4.0	4.0	10.4

Tannin and mimosine contents of the diets were calculated from the determined values in the LLM.

removal and drying at 60°C for 12h. The LLM used in diet 5.7 was similarly treated except that prior to heat treatment, the appropriate quantities of PEG and $Fe_2(SO_4)_3$ were well mixed in with the intention of assisting any interaction between these and the antinutritive factors in LLM. The mimosine supplemented diet (5.8) gave a mimosine content of 3.7g kg^{-1} of diet dry matter which was the same as that supplied by LLM in the LLM supplemented diets. The diets were formulated to provide about 12MJ of AME and 218g crude protein in each kg diet.

The analysis of the diets used in experiment 5 are presented in table 2.13.7.

2.13.6 Experiment 6.

Object

The object of this experiment was to attempt to improve the metabolisable energy of a sample of LLM (cv Peru, ex Malawi, 1977) with low mimosine content (Acamovic and D'Mello, 1981) and which has been shown to support adequate growth of chicks (D'Mello and Acamovic, 1982). Improvement of AME was attempted by supplementation of LLM with hemicellulase. Pre-incubation of the LLM, as a dough with the hemicellulase, resulted in an increase in the water soluble carbohydrate content from 20.6g kg^{-1} in the untreated LLM, to 41.5 g kg^{-1} in the hemicellulase treated material. The hemicellulase was added to the LLM in the same ratio as for the feeding experiment described below.

Two methods of enzyme supplementation were attempted. In one case the hemicellulase addition was to the diet containing the LLM (Table 2.13.8) while in the other the LLM was incubated (12h at 60°C) with a mixture of pectinase, cellulase and hemicellulase (6.2 cm^3, 0.8g and 8.3g respectively of each enzyme kg^{-1} LLM; obtained from Sigma Chemicals, London). The appropriate amount of enzyme was added to 2.4 dm^3 of distilled H_2O which was then mixed with 1.2kg of LLM prior to incubation.

The enzyme treated material was mixed with $Fe_2(SO_4)_3$ (7.5g kg^{-1} LLM) and dried in an oven at 60°C for 12h. This material was fed, alone, to twelve, three-week old chicks (weight range: 339-553g) for a period of two days. Prior to feeding the LLM, the birds were starved for 24h as they were for a further 24h period after the LLM was removed. At this time the excreta was collected for drying and analysis.

The diets supplemented with dry enzyme were supplied to the chicks as described earlier in this chapter.

Table 2.13.9 Composition of diets in experiment 7

ingredients (g kg^{-1})	diet No. 7.1	7.2	7.3	7.4
maize meal	429.9	145.6	141.6	137.6
soyabean meal	367.2	351.5	347.5	343.5
vegetable oil	10.0	10.0	10.0	10.0
sorghum	---	300.0	300.0	300.0
minerals & vitamins	44.9	44.9	44.9	44.9
(RS)methionine	3.0	3.0	3.0	3.0
glucose	80.0	80.0	80.0	80.0
cellulose	50.0	50.0	50.0	50.0
$Fe_2(SO_4)_3$	---	---	8.0	16.0
dietary components (by analysis; g kg^{-1} DM)				
ash	74.8	75.1	77.8	79.7
nitrogen	34.3	33.7	33.2	33.1
ether extract	32.0	31.6	34.0	39.2
fibre (TCA)	88.4	90.8	104.0	91.2
gross energy (MJ kg^{-1})	18.4	18.2	18.0	17.6
pH*	5.9	5.8	5.3	4.7
tannin£	---	6.1	6.1	6.1

£ calculated supplementary tannin content of the diet originating from the sorghum, the tannin content of which was determined using the method of the AOAC (1970).

* the pH was obtained by shaking the diet 3g of diet with 100g H_2O (sect. 2.13.1)

The composition of the sorghum used in experiment 7 is shown in Table 2.13.10

Table 2.13.10 The composition of the sorghum used in experiment 7

component	content (g kg^{-1} DM)
ash	20.7
nitrogen	15.7
ether extract	41.2
fibre (TCA)	20.4
gross energy (MJ kg^{-1})	19.1
tannin£	22.5

£ expressed as g gallotannic acid kg^{-1} DM and determined using the method of the A.O.A.C. (1970).

Table 2.13.11 Composition of diets used in Leucaena experiment 8.

diet No.	8.1
ingredients (g kg^{-1} diet)	
maize meal	809.1
soyabean meal	10.0
white fish meal	10.0
minerals & vitamins	44.9
vegetable oil	30.0
(RS) methionine	6.0

24g of mimosine were added to 8kg of the above diet to produce diet 8.2.

Table 2.13.12 Analysis of diets used in Leucaena experiment 8.

diet No.	8.1	8.2
component (g kg^{-1} DM)		
ash	69.9	70.0
nitrogen	24.7	24.1
ether extract	76.0	77.2
fibre (TCA)	19.2	19.6
gross energy (MJ kg^{-1})	18.4	18.4
mimosine£	---	3.4

£ calculated content from quantity added.

2.13.7 Experiment 7

Object

The purpose of this experiment was to evaluate the effect of Fe(III) on the antinutritional effects caused by the tannins present in the supplementary sorghum (obtained from the US Department of Agriculture, Washington, USA). The results may then be extrapolated to evaluate the effect, if any, of Fe(III) on the tannins present in the LLM diets in previous experiments. Diets (Table 2.13.9) were calculated to be iso-energetic (about 11 MJ kg^{-1}) and iso-nitrogenous (about 210 g CP kg^{-1}).

The composition of the sorghum used in experiment 7 is shown in Table 2.13.10

2.13.8 Experiment 8.

Object

The object of this experiment was to pair feed two diets, with and without mimosine, at a level lower than the ad lib consumption rate thus yielding information on the toxicity or antinutritional effects of mimosine on chicks. The experiment was also designed to test if mimosine ingestion above the acceptable daily intakes which have been proposed would cause deleterious effects. The AME and crude protein contents of the diets were calculated to be about 14 MJ kg^{-1} and 146 g kg respectively. The diets were designed to have protein levels close to the lower limits recommended for normal growth so that any deleterious effects of mimosine might be more evident. This effect has been demonstrated in rats fed diets containing supplementary amino acids (Harper, Becker and Stucki, 1966). Rats fed low protein diets containing disproportionately high levels of free individual amino acids have shown that excessive intakes of these amino acids retard growth more severely on the low protein diets than diets with higher protein contents (Harper, Becker and Stucki, 1966).

The intake level was determined by allowing the birds to eat the diet, without mimosine supplementation, on an ad lib basis for four days prior to starting the experiment. The daily food consumption for each chick was ascertained to be 81.7g. During the experiment each bird was provided with 70g diet each day for a period of 7 days. The birds were 24d old at the start of the experiment and weighed between 570-833g (mean= 784g) and two birds were allocated to each cage. The birds were fed 35g each in the morning and afternoon but otherwise treated as described previously. Diet compositions are shown below (Table 2.13.11) and were designed to be adequate in all nutrients.

Table 2.13.15 Composition of the diets used in experiment 9.

ingredients (g kg^{-1})	diet No. 9.1	9.2	9.3	9.4	9.5
maize meal	441.1	379.1	379.1	379.1	379.1
soyabean meal	243.0	165.0	165.0	165.0	165.0
white fish meal	100.0	100.0	100.0	100.0	100.0
glucose	121.0	120.3	124.1	131.2	135.0
mineral & vitamins	44.9	44.9	44.9	44.9	44.9
(RS) methionine	3.0	3.7	3.7	3.7	3.7
vegetable oil	20.0	20.0	20.0	20.0	20.0
jack beans[£]	---	140.0	140.0	140.0	140.0
glutamic acid	27.0	27.0	16.9	10.1	---
lysine. HCl	---	---	6.3	---	6.3
arginine. HCl	---	---	---	6.0	6.0
dietary components (by analysis; g kg^{-1} DM)					
ash	86.8	82.7	81.7	83.9	81.6
nitrogen	36.5	35.6	35.2	35.6	36.1
ether extract	44.8	45.2	44.8	46.4	45.6
fibre (TCA)	36.0	43.6	43.6	45.2	44.8
gross energy (MJ kg^{-1})	17.6	17.8	17.8	17.8	17.7
canavanine*	---	3.7	3.7	3.7	3.7

[£] autoclaved as described in the text
* calculated from the canavanine content of the autoclaved seed measured by HPLC.

The composition of the jack bean (after autoclaving) used in this experiment is shown below (Table 2.13.16).

Table 2.13.16 Composition of autoclaved jack beans used in experiment 9.

component	content (g kg^{-1} DM)
ash	22.0
nitrogen	49.8
ether extract	20.8
fibre(TCA)	113.6
gross energy (MJ kg^{-1})	18.7
canavanine (by HPLC)	25.1
canaline (by HPLC)	not detected

2.13.9 Experiment 9.

Object

The object of the experiment was to assess the effectiveness of arginine and/or lysine supplementation in alleviating the deleterious effects of feeding jack bean diets to chicks. It is well documented that arginine reduces the effects of the presence of canavanine (Rosenthal, 1977) and recent studies have demonstrated that arginine caused beneficial responses in chicks fed diets containing JB (D'Mello, Acamovic and Walker, 1985).

The jack beans used in this and other experiments were obtained from Mexico (FMVZ, University of Yucatan, Merida, Yucatan, Mexico). The beans were soaked in water for 24h at about 40°C and then minced to pass an aperture of 10mm prior to autoclaving at 134°C for 3min (Sybron/ Drayton Castle autoclave) using the porous cycle. The beans were then dried at 60°C for 3d and then ground to pass a 7mm aperture.

The untreated jack beans were determined to contain 2.5 g kg^{-1} of tannin on a dry matter basis. Tannin was determined using a modification of the AOAC (1970) method. The modification involved taking twice the recommended weight of the sample for analysis and also taking twice the recommended volumes of extract for titration.

Diets

Jack beans were included in the diets at the expense of soya bean and maize meal. Lysine and arginine were supplemented at the expense of glutamic acid. The diets were designed to be isonitrogenous and isoenergetic providing 230g kg^{-1} and 12.2 MJ kg^{-1} of CP and AME respectively of air dry diet. The composition of the diets used in this experiment is shown in table 2.13.15.

The composition of the jack bean (after autoclaving) used in this experiment is shown in Table 2.13.16).

2.13.10 Experiment 10.

Object

The object of this experiment was to investigate further the effects of supplementing jack bean diets with the canavanine and canaline analogues, arginine and ornithine as well as addition of lysine and glycine.

Table 2.13.17 Composition of the diets used in experiment 10.

ingredients (g kg^{-1})	diet No. 10.1	10.2	10.3	10.4	10.5
maize meal	441.1	379.1	379.1	379.1	379.1
soyabean meal	243.0	165.0	165.0	165.0	165.0
white fish meal	100.0	100.0	100.0	100.0	100.0
minerals & vitamins	44.9	44.9	44.9	44.9	44.9
glucose	121.0	120.3	124.1	135.0	135.7
jack beans[£]	---	140.0	140.0	140.0	140.0
vegetable oil	20.0	20.0	20.0	20.0	20.0
(RS) methionine	3.0	3.7	3.7	3.7	3.7
glutamic acid	27.0	27.0	18.9	2.0	---
lysine.HCl	---	---	---	---	1.3
arginine.HCl	---	---	---	6.0	6.0
ornithine.HCl	---	---	1.5	1.5	1.5
glycine	---	---	2.8	2.8	2.8
dietary components (by analysis g kg^{-1} DM)					
ash	74.1	75.6	73.0	70.2	72.7
nitrogen	39.9	40.4	39.8	39.6	39.1
ether extract	46.4	48.4	46.8	43.6	45.2
fibre (TCA)	29.2	37.2	38.8	37.2	39.6
gross energy (MJ kg^{-1})	18.4	18.3	18.4	19.2	18.3
canavanine[£]	---	3.5	3.5	3.5	3.5

[£] calculated from the content of jack beans.

The jack beans used in this experiment were prepared as for experiment 9 but then autoclaved at 121°C for 1h (Sybron/Drayton Castle autoclave) using the fluid cycle. After drying the beans were ground as described above and then reground. The composition of the jack beans used in this experiment is shown in Table 2.13.18.

Table 2.13.18 Composition of jack beans used in experiment 10.

component	content (g kg^{-1} DM)
ash	20.3
nitrogen	49.0
ether extract	16.4
fibre (TCA)	123.6
gross energy (MJ kg^{-1} DM)	19.1
canavanine (by HPLC)	24.3
canaline (by HPLC)	not detected

Diets

The composition of the diets are shown in Table 2.13.17. The supplementary amino acids were added at the expense of glutamic acid. The AME and crude protein contents of the diets were calculated to be about 12 MJ kg^{-1} and 230g kg^{-1} respectively and they were designed to contain an adequate source of other nutrients.

The jack beans used in this experiment were prepared as for experiment 9 but then autoclaved at 121°C for 1h (Sybron/Drayton Castle autoclave) using the fluid cycle. After drying the beans were ground as described above and then reground. The composition of the jack beans used in this experiment is shown in Table 2.13.18.

2.13.11 Experiment 11.

Object

There were a number of objectives in conducting this experiment:

a. To examine the effect of dietary inclusion of germinated jack beans on chicks.
b. To examine the effect of drying temperature of the autoclaved beans on chicks.
c. To examine the effect of the dietary inclusion of canavanine and the resultant canavanine extracted residue, on chicks.
d. Simultaneously a comparison between different methods of soaking would be made.

Jack beans were germinated for 3 and 5 days on trays lined with cotton wool. The cotton wool was maintained in a moist condition during the germination period. The outer seed coat of the bean had a hole made in it prior to placing in the tray for germination. Germination was achieved at ambient temperature at the FMVZ in Merida, Mexico. At the end of the germination period the beans were removed from the trays and dried at 60°C in an oven. The dried beans were then ground (1mm aperture) and mixed with water in a tray until a thick dough was produced. The dough was then autoclaved at 121°C for 1h prior to drying for 3 days in an oven at 60°C. The resultant friable mass was ground (twice) to pass a 7mm aperture producing a granular powder.

Ungerminated jack beans were treated as described in experiment 10 and some of the autoclaved beans dried at 60°C while another portion was dried at 100°C.

Table 2.13.19 Composition of the diets used in experiment 11.

ingredients ($g\ kg^{-1}$)	Diet No. 11.1	11.2	11.3	11.4	11.5	11.6	11.7	11.8	11.9	11.10
maize meal	441.1	379.1	343.4	343.4	379.1	437.2	379.1	343.4	343.4	343.4
soyabean meal	270.0	192.0	108.0	108.0	192.0	270.0	192.0	108.0	108.0	108.0
glucose	121.0	120.3	100.0	100.0	120.3	121.0	120.3	100.0	100.0	100.0
white fish meal	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
minerals & vitamins	44.9	44.9	44.9	44.9	44.9	44.9	44.9	44.9	44.9	44.9
vegetable oil	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
(SR)methionine	3.0	3.7	3.7	3.7	3.0	3.0	3.7	3.7	3.7	3.7
jack bean (a)	---	140.0	---	---	---	---	---	---	---	---
jack bean (b)	---	---	280.0	---	---	---	---	---	---	---
jack bean (c)	---	---	---	280.0	---	---	---	---	---	---
jack bean (d)	---	---	---	---	140.0	---	---	---	---	---
canavanine	---	---	---	---	---	3.9	---	---	---	---
jack bean (e)	---	---	---	---	---	---	140.0	280.0	---	---
jack bean (f)	---	---	---	---	---	---	---	---	280.0	---
jack bean (g)	---	---	---	---	---	---	---	---	---	280.0

(a) ground, made into a dough, autoclaved and dried at 60°C

(b) germinated (3d), ground, made into a dough, autoclaved and dried at 60°C.

(c) germinated (5d), ground, made into a dough, autoclaved and dried at 60°C.

(d) residue after extraction of canavanine, autoclaved and dried at 60°C.

(e) ground, made into a dough, autoclaved and dried at 100°C.

(f) whole beans soaked for 24h in tap water at 40°C, minced, autoclaved and dried at 60°C.

(g) whole beans soaked for 24h in tap water at 40°C, minced, autoclaved and dried at 100°C.

Table 2.13.20 Composition, by analysis, of the diets used in experiment 11.

component (g kg^{-1} DM)	diet No. 11.1	11.2	11.3	11.4	11.5	11.6	11.7	11.8	11.9	11.10
ash	78.1	77.1	78.4	77.6	77.9	79.1	80.0	74.5	72.6	75.1
nitrogen	38.1	38.1	35.4	38.1	35.7	40.4	38.1	37.4	36.5	37.4
ether extract	50.0	51.6	51.2	47.2	50.0	51.6	50.4	49.6	50.0	53.2
fibre (TCA)	38.4	44.8	47.6	50.4	42.8	38.8	44.8	51.2	53.2	50.0
gross energy (MJ kg^{-1})	18.5	18.5	18.2	18.3	18.3	18.8	18.2	18.4	18.4	18.4
canavanine[£]	---	2.9	9.5	8.8	0.33	4.0^	2.6	5.2	5.8	2.8

£ calculated from the determined (by HPLC) values in the jack bean
^ determined value in the diet.

Table 2.13.21 Composition of jack beans used in experiment 11.

component (g kg^{-1} DM)	jack bean treatment a	b	c	d	e	f	g
ash	26.5	29.0	31.4	13.0	27.5	21.4	25.5
nitrogen	44.2	41.7	43.1	35.1	45.4	42.4	43.7
ether extract	17.2	18.8	13.2	15.2	18.8	17.2	18.8
fibre (TCA)	103.6	102.8	112.8	118.8	106.8	111.6	110.4
gross energy (MJ kg^{-1})	18.8	18.4	18.5	18.7	18.5	19.0	18.9
canavanine	20.5	33.5	30.7	2.2	18.3	20.6	9.8

jack bean treatments prior to analysis:
(a) ground, made into a dough, autoclaved & dried at 60°C.
(b) germinated (3d), ground, made into a dough, autoclaved & dried at 60°C.
(c) germinated (5d), ground, made into a dough, autoclaved & dried at 60°C.
(d) residue after extraction of canavanine, autoclaved & dried at 60°C.
(e) ground, made into a dough, autoclaved and dried at 100°C.
(f) whole beans soaked in tap water at 40°C for 24h, minced, autoclaved and dried at 60°C.
(g) whole beans soaked in tap water at 40°C for 24h , minced, autoclaved and dried at 100°C.

Table 2.13.22 The composition of the diets used in experiment 12.

ingredient (g kg^{-1})	diet No. 12.1	12.2	12.3	12.4	12.5
maize meal	440.5	379.1	379.1	379.1	379.1
soyabean meal	222.0	143.4	143.4	143.4	143.4
white fish meal	100.0	100.0	100.0	100.0	100.0
minerals & vitamins	44.9	44.9	44.9	44.9	44.9
vegetable oil	20.0	20.0	20.0	20.0	20.0
(RS) methionine	3.0	3.7	3.7	3.7	3.7
glucose	121.0	120.3	129.1	137.9	146.7
jack bean*	---	140.0	140.0	140.0	140.0
glutamic acid	48.6	48.6	32.4	16.2	---
arginine.HCl	---	---	3.6	7.3	10.9
lysine.HCl	---	---	3.8	7.5	11.3
dietary components (by analysis; g kg^{-1} DM)					
ash	72.0	68.7	71.3	71.3	71.7
nitrogen	39.3	39.3	38.7	37.9	38.1
ether extract	51.2	50.4	50.0	50.4	48.4
fibre(TCA)	31.2	38.8	35.6	36.0	36.4
gross energy (MJ kg^{-1})	18.0	17.9	18.0	18.0	18.1
canavanine[£]	---	2.9	2.9	2.9	2.9

[£] calculated from determined value in jack beans used.

* jack beans ground, made into a dough, autoclaved & dried at 60°C.

Some ungerminated jack beans were also ground (1mm aperture), mixed with water until a dough was produced and then autoclaved at 121°C for 1h. The resultant mass was dried at 60°C and ground twice (7mm aperture). Another portion was similarly treated but dried at 100°C prior to grinding.

A preliminary experiment showed that drying finely ground jack bean, which had been mixed with water to give a dough, at 100°C for 18h produced a 20% reduction in the canavanine content as measured by the extraction procedure and HPLC technique described earlier in this chapter (sect. 2.4).

Canavanine was extracted from finely ground jack beans as described earlier (section 2.6.1). The residue, in the polypropylene centrifuge bottles was autoclaved for 1h at 121°C. The resultant material, which included a solid gelatinised mass, was removed from the bottles and dried at 60°C prior to grinding (7mm aperture).

Diets

Each of the treated jack bean fractions were incorporated into the diets as shown in Table 2.13.19. Calculated AME and crude protein contents were about 12.5MJ kg^{-1} and 225 g kg^{-1} on an air dry basis respectively. They were otherwise designed to be adequate in all other nutrients.

The analytical data for the jack beans used in this experiment are presented in Table 2.13.21.

During the chromatographic determination of canavanine in the beans, qualitative examination of the chromatograms readily showed that there was a large increase in the concentration of free amino acids in the extracts from germinated beans compared with extracts from ungerminated beans.

2.13.12 Experiment 12.

Object

This experiment was conducted to further investigate the effects of arginine and lysine on chicks fed jack bean diets. The composition of the diets are presented in Table 2.13.22.

Diets

The diets were deigned and prepared to be similar to those used in experiment 10 with the exception that a larger amount of glutamic acid was required to

Table 2.13.24 Composition of the diets used in experiment 13.

ingredient (g kg^{-1})	diet No. 13.1	13.2	13.3	13.4	13.5
glucose	450.0	250.0	50.0	250.0	50.0
white fish meal	50.0	50.0	50.0	50.0	50.0
soyabean meal	422.1	422.1	422.1	422.1	422.1
minerals & vitamins	44.9	44.9	44.9	44.9	44.9
(RS) methionine	3.0	3.0	3.,0	3.0	3.0
vegetable oil	30.0	30.0	30.0	30.0	30.0
lupins (uncooked)	---	200.0	400.0	---	---
lupins (cooked)	---	---	---	200.0	400.0
dietary components (by analysis g kg^{-1} DM)					
ash	73.4	85.9	92.2	80.0	88.3
nitrogen	36.7	48.1	61.4	49.8	60.7
ether extract	38.8	57.2	77.2	59.2	87.6
fibre (TCA)	29.2	52.8	77.2	59.2	87.6
gross energy (MJ kg^{-1})	17.7	18.7	19.8	18.9	19.9

Table 2.13.25 Composition of diets in experiment 14.

ingredient (g kg^{-1})	diet No. 14.1	14.2	14.3	14.4	14.5
maize meal	429.8	382.7	381.2	382.7	382.7
soyabean meal	367.2	82.4	82.4	82.4	82.4
vegetable oil	10.0	10.0	10.0	10.0	10.0
white fish meal	15.0	15.0	15.0	15.0	15.0
minerals & vitamins	44.9	44.9	44.9	44.9	44.9
(RS) methionine	3.0	5.0	5.0	5.0	5.0
glucose	80.0	10.0	10.0	10.0	10.0
cellulose	50.0	50.0	50.0	50.0	50.0
lupin (a)	---	400.0	400.0	---	---
lupin (b)	---	---	---	400.0	---
lupin (c)	---	---	---	---	400.0
dry enzyme mix*	---	---	1.5	---	---
dietary component (by analysis g kg^{-1} DM)					
ash	74.8	72.4	71.8	68.9	69.0
nitrogen	34.3	40.7	35.7	36.5	37.2
ether extract	32.0	60.8	62.4	62.4	60.4
fibre (TCA)	88.4	117.2	117.6	112.8	108.0
gross energy (MJ kg^{-1})	18.4	19.1	19.3	19.6	19.4

a untreated lupin seed
b incubated with enzyme mixture at a level of 3.8g kg^{-1} of lupin.
c incubated with enzyme mixture at a level of 7.6g kg^{-1} of lupin.
* dry enzyme included pectinase, a-amylase, amyloglucosidase and pentanosidase mixture added at a level of 3.8g kg^{-1} lupin.

compensate for the increased quantities of arginine and lysine added to the diets in this experiment.

The analysis of the diets are presented in Table 2.13.22 while the analysis of the jack beans used in this experiment are shown in table 2.13.21 (treatment a).

2.13.13 Lupin experiment 13.

Object

The objectives in this experiment were to obtain AME values of locally grown seeds from lupins (*Lupinus albus* cv. Vladimir [Kievskji mutant]). The effect of heat treatment of the seeds on their AME was also intended. The seeds were grown in the Berwick on Tweed area of Scotland (a gift of K. Wilson, Berwick on Tweed) and either fed cooked or uncooked as part of a semi-purified diet. There was no apparant fungal infestation of these seeds therefore the likelyhood of the presence of mycotoxins being present was small (sect. 1.11.1). It is worth mentioning that the other plant materials used during the course of the work presented here also did not appear to be affected by fungus.

The lupin seed was substituted at the expense of glucose and AME values calculated by the method described by other workers (D'Mello and Whittemore, 1975). This method of determination of AME has been discussed in detail recently (Pesti and Ware, 1986).

The cooked lupins were prepared by soaking the whole seed in water at 40°C for 6h then autoclaved at 121°C in a similar manner to jack beans. The seeds were dried in an oven at 60°C for 48h prior to grinding twice (through a 7mm aperture).

Untreated lupins were ground twice (7mm aperture) prior to inclusion in the diets (Table 2.13.24). The analysis of the lupins used in this experiment is given below (Table 2.13.27).

Diets

The reference diet (13.1; Table 2.13.24) was designed to be adequate in all essential nutrients and to supply about 218 g CP and 11.9 MJ AME kg^{-1} air dry diet.

Table 2.13.27 Composition of lupins used in experiments 13 and 14.

component ($g\ kg^{-1}$ DM)	lupin treatment untreated	cooked	incubated	incubated
level of enzyme added ($g\ kg^{-1}$)	---	---	3.8	7.6
ash	36.0	37.6	37.5	40.6
nitrogen	54.1	58.4	56.6	57.7
ether extract	96.4	102.8	86.8	92.0
fibre (TCA)	106.4	124.0	120.0	112.4
gross energy ($MJ\ kg^{-1}$ DM)	20.2	20.9	19.2	21.7
tannins*	1.9	0.90	ND	ND

* Determined using the AOAC method, (1970).
ND not determined.

2.13.14 Experiment 14.

The supply of lupins used in this experiment were the same as those used in experiment 13 (section 2.13.13).

Object

The objectives of this experiment were to attempt to improve the nutritional value, particularly AME, of lupins by supplementation with enzymes. Two methods of supplementation were attempted. The first was to add the enzyme mixture in dried form, to the diet. The second was to mix the enzymes with a wet mixture of the ground lupins and incubate prior to inclusion in the diets.

The enzymes used were pectinase (EC 3.2.1.15), a-amylase (EC 3.2.1.1), amyloglucosidase (EC 3.2.1.3) and pentanosidase (EC 3.2.1.7.8) and were supplied by ABM Chemicals Ltd, Stockport, Cheshire, U.K.

From the information given by ABM Ltd pectinase was of fungal origin having high pectolytic activity. The useful pH range of the enzyme was given to be between pH 2.5-6.0 at a temperature of 50°C. Amylase was of bacterial origin having an effective pH range of 4.5-8.5 at 60°C. Pentanosidase was obtained from Trichoderma viride and determined to have an optimum pH of 5.5 at 55°C. Amyloglucosidase was of fungal origin having a working pH range of 3.5-6.0 and an optimum temperature of about 65°C.

The enzyme treated lupin was prepared by mixing either 4.8 or 9.6g of each enzyme in $2dm^3$ tap water at about 30°C. The well mixed enzyme suspension was equally divided between two lots of 2.5kg of ground lupin seed in polyethylene containers. A further $2.2dm^3$ of tap water was added and the resultant dough thoroughly mixed. The containers were then sealed and placed in an incubator at 60°C for 24h after which they were removed and dried in trays in an oven for 48h. The dried product was ground (7mm aperture) prior to inclusion of the resultant brown (Demarara sugar-like smell and appearance) into the diets. The composition of the lupins prepared in this way are presented in Table 2.13.27.

Diets

The diets (Table 2.13.25) were designed to have an AME of about 11MJ kg^{-1} and a protein content of about 200g kg^{-1} of diet as fed and otherwise have an adequate supply of all other essential nutrients.

CHAPTER 3

RESULTS

3.1 Analysis of mimosine and 3-hydroxy-4(1H)-pyridone.

3.1.1 Analysis using HPLC and UV detection.

Plots of concentration of mimosine and 3,4-DHP against peak area (measured manually and calculated as the peak width at half height multiplied by the height and expressed in mm^2) are shown in Fig. 3.1.1 The concentration ranges vary from 6.25uM to 0.25mM for mimosine and 12.5uM to 0.25uM for 3,4-DHP. Each standard was analysed in triplicate at their lambda max wavelenghths of 278nm and 269nm for mimosine and 3,4-DHP respectively. The results indicate excellent linearity of response and precision over the working ranges at the different wavelenghths and sensitivities. The linear regression equations, correlation coefficients and standard errors for each response curve for loaded quantities in the range 0.125 to 5nmol are shown in the legend of Fig. 3.1.1.

Chromatograms of a standard mixture of mimosine and 3,4-DHP, a 6M HCl extract of LLM, a serum sample from a chick fed LLM and a serum sample from a chick fed a diet free of LLM are presented (Fig. 3.1.2 a, b, c and d respectively). Elution of mimosine and 3,4-DHP occurs within 12min of injection. Chromatograms c and d, when compared with each other, (Fig. 3.1.2) show an interesting difference in profile over the first 4min. The chromatogram (Fig. 3.1.2b) obtained from an LLM extract has similar characteristics, during the first 4 min , to that (Fig. 3.1.2c) from the serum of chicks fed the LLM based diet. The earlier part of the chromatogram, obtained from the chick serum from LLM fed birds is much more congested than the serum from birds fed LLM free diet.

From the chromatograms (Fig. 3.1.2 a and b) it is obvious that baseline resolution of mimosine and 3,4-DHP is almost achieved. The mean resolution (Rs) obtained from nine samples was 1.62 with a relative standard deviation (RSD) of 0.0654 (RSD= SD/mean). The nine samples used for the calculation of Rs consisted of LLM extracts, excreta extracts, standard mixture and spiked serum, chromatographed on different days. The variation of sample and the time lapse between analysis is likely to account for the fairly high RSD. The resolution was calculated as follows: Rs= 2(tr.3,4-DHP-tr. mimosine)/W.3,4-DHP+W.mimosine) where tr.3,4-DHP and tr.mimosine are the retention times of 3,4-DHP and mimosine respectively and W.3,4-DHP and W.mimosine are the

Fig. 3.1.1 HPLC response curves for mimosine (a,b) and 3-hydroxy-4 (1H)-pyridone (c,d) at 278 and 269 nm, respectively. Concentration (mM) plotted on abscissa, peak area (mm^2) on ordinate. (a) 0.05 a.u.f.s.: y = 3271.0x + 6.374; standard error (S.E.) = 10.3; correlation coeff. (R) = 0.999. (b) 0.01 a.u.f.s.; y = 14,738x - 15.208; S.E. = 11.2: R = 0.999. (c) 0.02 a.u.f.s.: y = 4486.6x - 0.77; S.E. = 19.3; R = 0.999. (d) 0.01 a.u.f.s.: y = 8513.5x + 3.57: S.E. = 10.00: R = 1.000.

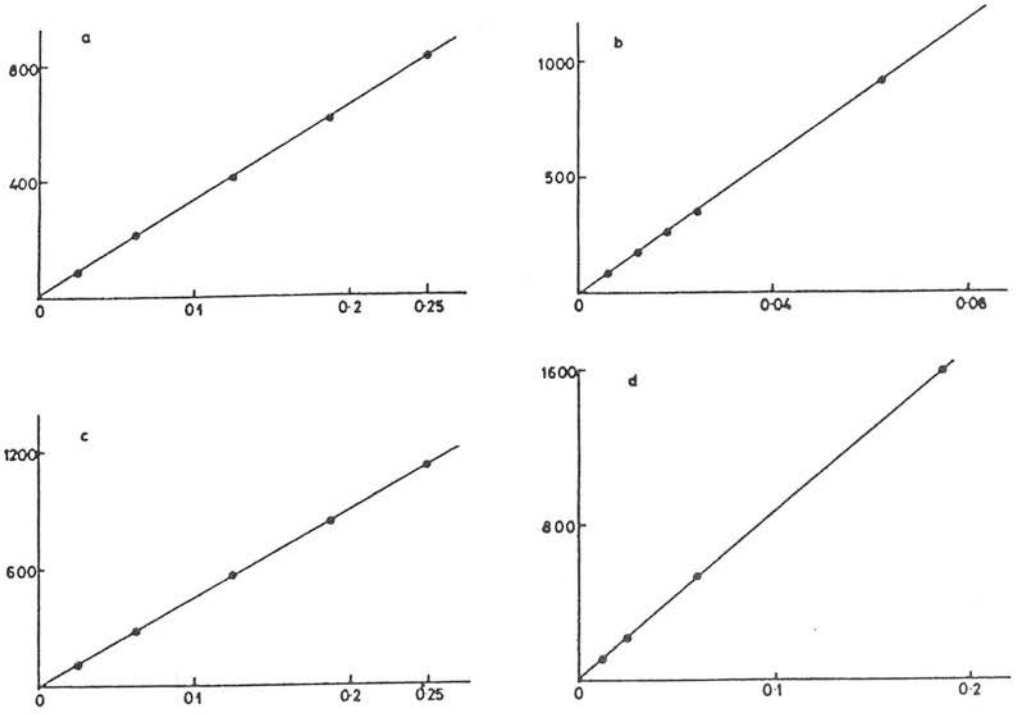


Fig. 3.1.2 Typical chromatograms of (a) a standard solution of mimosine and 3-hydroxy-4 (1H)-pyridone; (b) a *Leucaena* leaf meal (LLM) extract; and serum samples from chicks fed (c) an LLM diet and (d) a diet without LLM. Peaks: 1 = mimosine; 2 = DHP. Flow-rate of pH 2.25 buffer, 1.8 cm^3 min^{-1} ; detector 269 nm and 0.02 a.u.f.s.

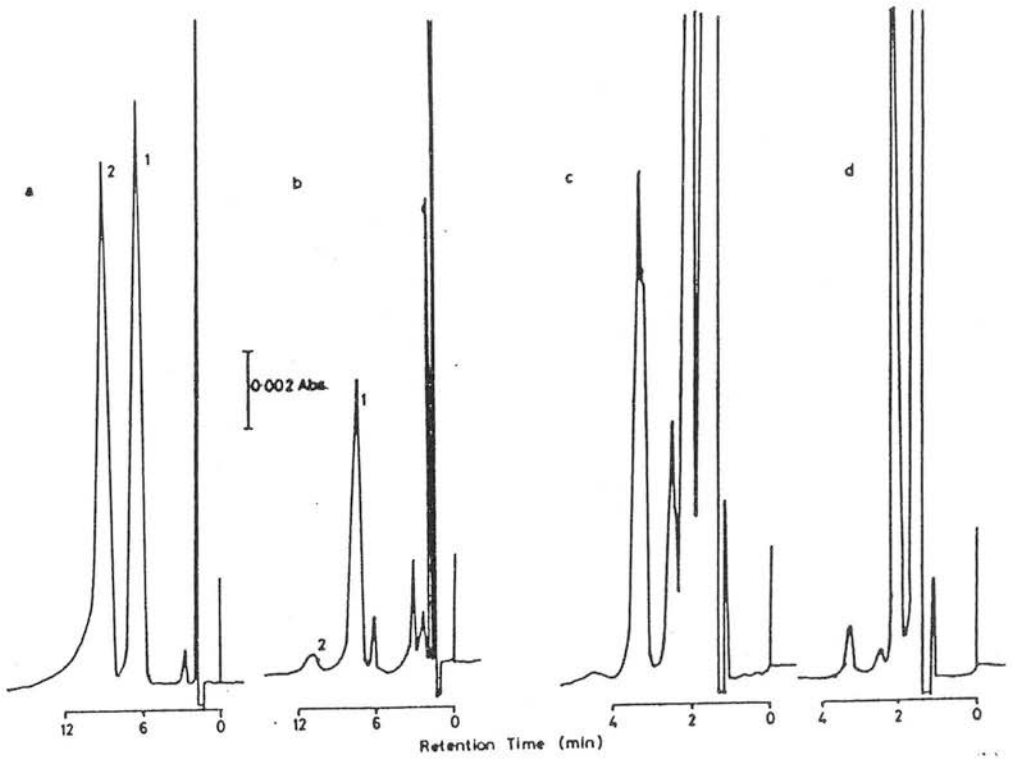


Table 3.1.1 Mimosine content of Leucaena leucocephala leaf meal (LLM), seeds (LS) and chick excreta, determined using ion exchange chromatography (IEC) and HPLC with UV detection.

sample	mimosine concentration (g kg^{-1} dry matter)		HPLC/IEC
	IEC*	HPLC (RSD)	
LLM sample 1 (cv Peru)	24.27	24.73 (0.028)*	1.019
LLM sample 2 (cv Peru)	10.26	10.42 (0.025)**	1.016
LLM sample 3 (cv Peru)	23.28	23.76 (0.011)***	1.021
LS sample 1	67.54	67.95 (0.005)‡	1.006
LS sample2	73.19	73.93 (0.001)‡	1.010
chick excreta (sample 1)†	3.63	3.43 (0.008)‡	0.945
chick excreta (sample 2)†	1.11	1.01 (0.007)‡	0.910

* Mean of duplicate analysis of three lots of the sample.
** Mean of duplicate analysis of six lots of the sample.
*** Mean of four analysis of two lots (two at 269nm and two at 278nm).
‡ Mean of triplicate analysis of one lot of each sample.
† From chicks fed LLM diets.
* method of Acamovic and D'Mello, 1981.

Table 3.1.2 Recovery of 3-hydroxy-4(1H)-pyridone (3,4-DHP) from Leucaena leaf meal (LLM) and chick excreta using HPLC (measured at 269nm).

sample	3,4-DHP content (g kg^{-1} DM) analysis	expected	recovery (RSD)
LLM + 3,4-DHP*	3.084	3.069	1.005 (0.026)
LLM + 3,4-DHP**	5.475	5.912	0.093 (0.056)
LLM + 3,4-DHP**	8.484	9.024	0.981 (0.055)
chick excreta + 3,4-DHP **	5.197	4.974	1.045 (0.013)

* 3,4-DHP solution added to three LLM extracts prior to analysis.
** Crystalline 3,4-DHP was added to powdered sample prior to extraction. Three samples were taken for extraction.

Table 3.1.3 Recoveries of additional mimosine and 3-hydroxy-4(1H)-pyridone (3,4-DHP) from chick serum using HPLC.

	serum sample A*	B**	C**
mimosine concentration (nmol cm^{-3})	50.8	34.3	22.2
mimosine expected (nmol cm^{-3})	62.5	62.5	31.25
mimosine recovery (RSD)	0.813(0.068)	0.549(0.031)	0.711(0.063)
3,4-DHP concentration (nmol cm^{-3})	72.1	102.3	47.2
3,4-DHP expected (nmol cm^{-3})	125	125	62.5
3,4-DHP recovery (RSD)	0.577(0.028)	0.818(0.026)	0.755(0.016)
combined recovery of mimosine & 3,4-DHP	0.695	0.684	0.733

* Values are the means of triplicate analyses of three samples; sulphosalicylic acid used as protein precipitant.
** Values are the means of duplicate analyses of three samples; phosphotungstic acid used as protein precipitant.

base widths of 3,4-DHP and mimosine respectively. The base widths were obtained by drawing tangents to the peaks at half heights. The k' values of mimosine and 3,4-DHP were 3.63 (+-0.33) and 5.56 (+-0.49) respectively as determined using the same nine chromatograms used for the determination of Rs.

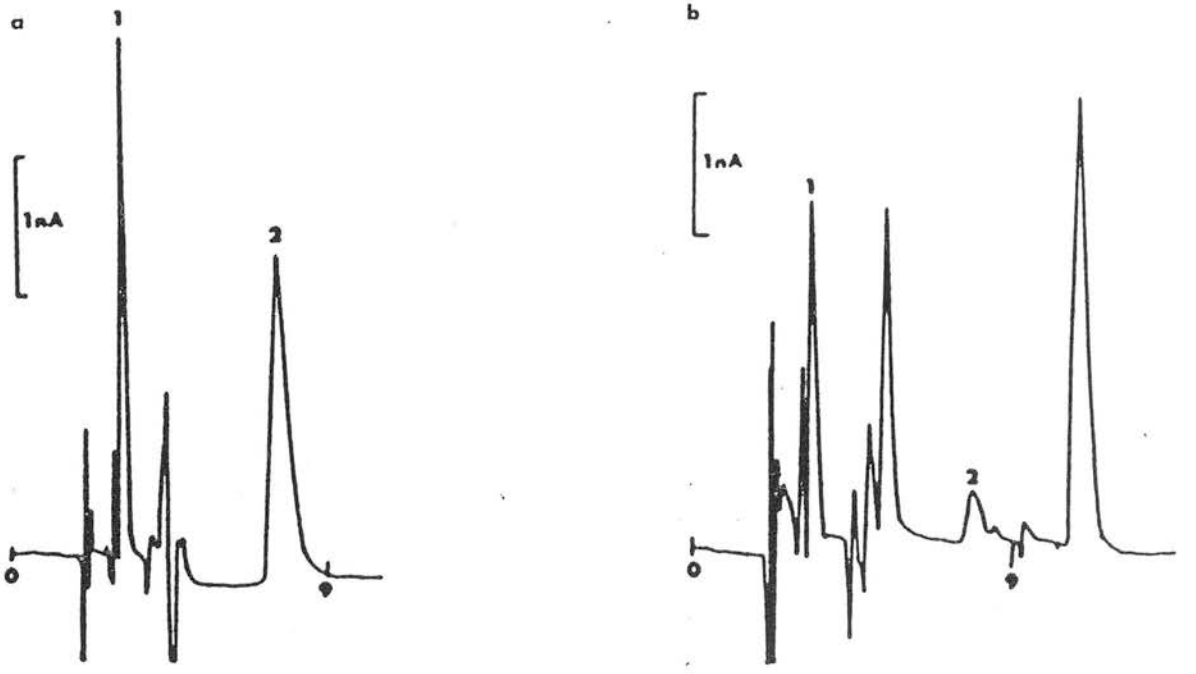
It should be noted that a small peak was apparent at the base of the mimosine peak during the analysis of some samples; its absence or presence did not affect the quantitation of mimosine nor 3,4-DHP as reflected in the recoveries of supplemental mimosine and 3,4-DHP (Tables 3.1.1 and 3.1.2). The mimosine content of LLM, *leucaena* seeds and excreta as determined using HPLC correlate well with the values obtained using IEC (Table 3.1.1). Although the values for the excreta are slightly lower by HPLC than with IEC there is no significant ($P>0.05$) differences between the results obtained using IEC and HPLC when compared using a paired t-test (Snedecor and Cochran, 1967). The RSD of the values, determined using HPLC, is variable and largest when different extracts of the same sample were analysed. The variation, however, is within acceptable bounds for analyses of this type (Snyder and van der Wal, 1981). The good agreement between the results obtained using IEC and HPLC with detection at 278 and 269nm is indicative that there is little or no interference using HPLC.

Recovery of additional 3,4-DHP from LLM and chick excreta (Table 3.1.2) is approximately quantitative although RSD values are somewhat higher than those for mimosine.

Surprisingly neither mimosine nor 3,4-DHP were detected in the serum of chicks fed LLM diets. Recovery of added mimosine and 3,4-DHP from serum was poor (Table 3.1.3) indicating that substantial losses of these occur during sample preparation. Recovery of mimosine was highest when SSA was used as the precipitant and the mimosine and 3,4-DHP levels were 62.5 and 125nmol cm^{-3} respectively although precision of analysis was poor. Precision of analysis of 3,4-DHP, on the other hand, was good but recovery was low, when SSA was used as the protein precipitant. Peak splitting was observed after the analysis of about 12 samples treated with SSA. The problem was overcome by replacing the top 1-2mm of column packing material. Peak splitting did not occur when PTA was used as the protein precipitant.

The use of PTA, as the protein precipitant, caused lower recoveries of mimosine but higher recoveries of 3,4-DHP than SSA (Table 3.1.3). Recovery of mimosine was higher when the concentration of mimosine in the sample was lowest. The total molar recovery of mimosine and 3,4-DHP using either SSA or PTA as the precipitant were almost identical. It was considered that one of the reasons for

Fig. 3.1.3 Typical chromatograms of (a) a standard mixture of mimosine (1) and 3-hydroxy-4 (1H) - pyridone (2) and (b) an extract of *Leucaena leucocephala*



Conditions
Eluent: pH3 buffer, flow rate: 1 cm^3 min^{-1}, column; 4.6 x 250 mm Biophase 5 µm ODS, detection: ECD.

the poor recoveries may have been ion pairing of the molecules with the precipitants since complete recovery of standards and extracts was achieved without addition of the SSA or PTA. It seemed reasonable that if ion pairing had occurred then some proportion of the analytes may be eluted with the precipitant with minimal retention since both SSA and PTA are only slightly retained. This premise was tested by adding either SSA or PTA to a standard solution of mimosine and 3,4-DHP (0.125mM and 0.25mM respectively in 0.1M HCl). Analysis of these standards after passing through Sep-Pak cartridges confirmed that losses did occur. Recoveries of mimosine and 3,4-DHP were 0.86 and 0.88 respectively.

Column efficiency remained approximately constant throughout the analysis of about 900 samples as did retention times. It was necessary to replace the top 1-2mm of packing material on a number of occasions and also to clean the column by pumping with aqueous methanol (7+3 methanol:H_2O).

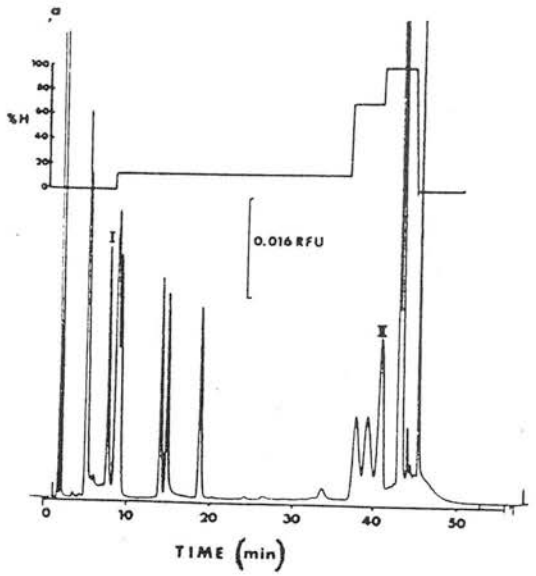
The HPLC method with UV detection was used for the analyses of mimosine in the LLM and LS samples used in the animal experiments. The method was also effective in quantifying mimosine and 3,4-DHP in the excreta of chicks fed diets containing LLM.

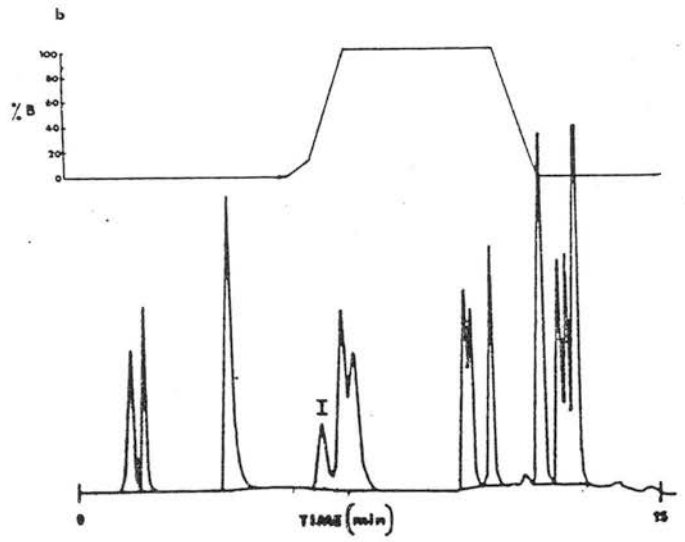
3.1.2 Electrochemical detection.

Chromatograms obtained from a standard mixture of mimosine and 3,4-DHP and a LLM extract, obtained using ECD are shown in Fig. 3.1.3. Both analytes elute within 9min of injection and are well resolved from each other although the mimosine peak is close to an early eluting ghost peak. Peak asymmetry was relatively low (0.5) but was the same when the column was used for catecholamine analysis.

A linear response was obtained when concentration (0.1-12.5uM) of mimosine and 3,4-DHP were plotted against peak area (R=1.000). The increased sensitivity using EC rather than UV detection allows 'on column' levels as low as 2pmol to be routinely measured thus permitting an increase in sensitivity of three orders of magnitude. A notable difference between the chromatogram of the standards and the LLM extract is the extra peaks in the extract. These are likely to be due to polyphenolics or tannins in the extract.

Fig. 3.2.1 Chromatograms of the OPA derivatives of a standard mixture of amino acids including canavanine (I) and canaline (II)





a: Conditions as in table 2.5.2; column: Shandon (5 x 160 mm) packed with Spherisorb ODS 2 (dp = 5 µm)

b: Conditions as in table 2.5.2; column: Shandon (5 x 100) packed with C_{18} Hypersil (dp = 3 µm): Detection: fluorimetry.

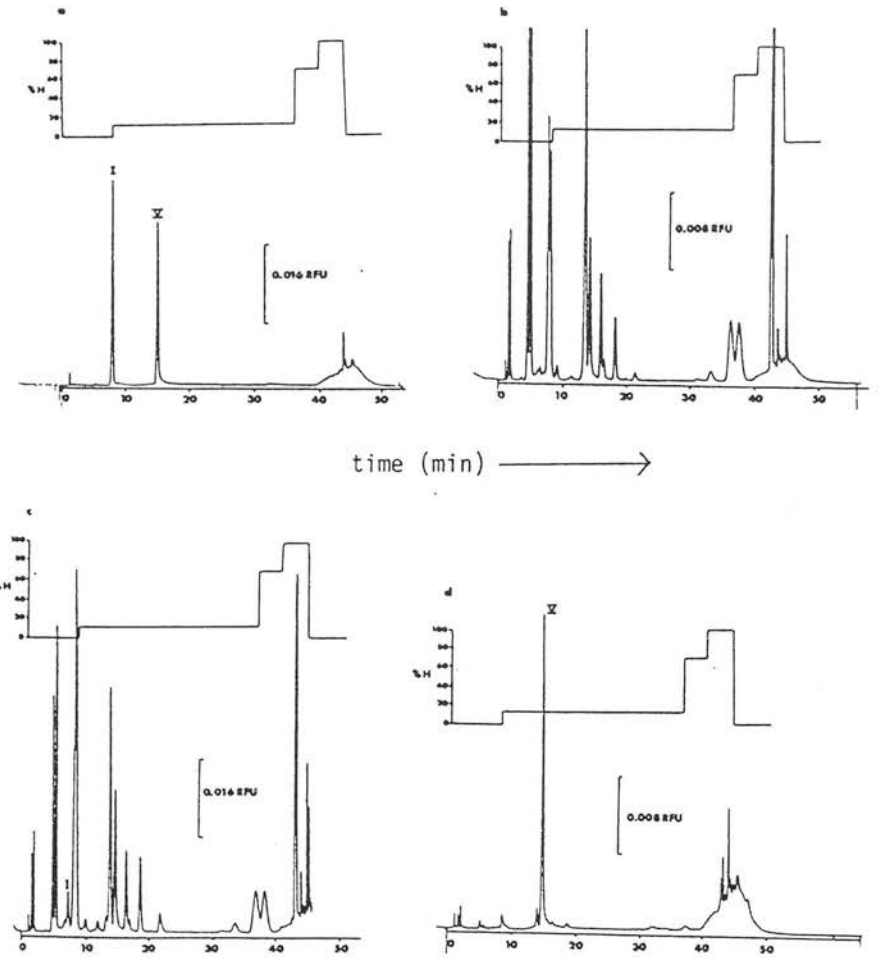
3.2 Analysis of canavanine and canaline.

Chromatograms of a standard mixture of amino acids including canavanine and canaline are shown in Fig. 3.2.1. Canavanine (I) and canaline (II) were resolved from other amino acids in the mixture during the 50min run using 5um Spherisorb in a 160mm Shandon column (Fig. 3.2.1a) while the 15min run using a 100mm column containing Hypersil (Fig 3.2.1b), resolved canavanine (I) from the other amino acids but not canaline. The amount of each amino acid loaded was 75pmol except for canaline which was 270pmol. It should be noted that column selectivities are comparable as witnessed by the similarity in the chromatograms. It should also be noted that Fig. 3.2.1b was obtained from the Trio data handling device which attenuates the peaks so that the largest peak is produced on the full scale, while Fig. 3.2.1a was obtained from the recorder chart output. The major difference between the chromatographic methods is the time taken for each analysis. Linear responses were obtained (R=0.998) when amount loaded (1-300 pmol) was plotted against peak area for both canavanine and canaline.

The mean k' values for canavanine and canaline peaks were 6.6 (RSD= 0.054) and 34.8 (RSD= 0.041). These values were obtained on two separate days at ambient temperature using the 160mm column packed with 5um Spherisorb. Resolution of both peaks was maintained on both days as well as on other days. Chromatograms of a jack bean extract, a serum from chicks fed a JB diet (140g kg^{-1}) and a jack bean free diet, as well as an excreta extract from chicks fed JB are shown (Fig. 3.2.2 a, b, c, d respectively). It is clear that canavanine but no canaline was present in the JB extract. The peak which has a retention time of about 15min.(IV; Fig. 3.2.2) is due to the production of an artefact from the OPA reagent. It was found that this peak increased in size with increasing age of the OPA reagent. The artefact peak did not interfere with the canavanine or canaline peaks but did interfere with the peaks produced by other amino acids. Other than the artefact peak and the canavanine peak the jack bean extract was relatively free from other OPA reactive compounds. Germinated seeds, however, produced a considerable number of peaks presumably due to proteolysis during germination (Palmer, McIntosh and Pusztai, 1973).

Comparison of the chromatograms of serum from chicks fed diets containing JB or without JB (Fig. 3.2.2 a & b), shows that there is a peak attributable to canavanine with the former while it is absent from the latter. A canaline peak was not detected in any of the serum samples tested. Modification of the derivatisation procedure so that 100ul of the acetonitrile pretreated sample was added to 100ul of OPA solution, as well as working with a detector sensitivity

Fig. 3.2.2 Typical chromatograms of jack bean extract (a),serum from a chick fed jack beans (b),serum from a chick fed a jack bean free diet (c) and an excreta extract from chicks fed a jack bean diet (d).



I: canavanine; V: reagent artefact peak.
Conditions as in table 2.5.2 column: Shandon (5 x 160 mm) packed with Spherisorb ODS (dp = 5 µm).
Detection: fluorimetric detection of the OPA derivatives.

Table 3.2.1 Canavanine concentration (g kg^{-1}) in a sample of jack beans (as received) measured using HPLC, ion exchange chromatography (IEC) and colorimetry.

method of analysis	HPLC	colorimetry*	HPLC	IEC
method of sample preparation	(H_2O extraction)		(HCl hydrolysis)	
canavanine (g kg^{-1})	38.3	38.1	36.0	35.9
RSD	0.043	0.018	0.026	0.073
n	4	6	4	6

* by colorimetric examination of the pentacyanoammine ferrate (III) complex. Description of the methods used is given section 2.4.

Table 3.2.2 Recovery of added canavanine and canaline from jack beans by extraction with water.

	canavanine		canaline
method of analysis	HPLC	colorimetry*	HPLC
recovery (g g^{-1} added)	0.984	0.970	0.998
RSD	0.076	0.056	0.018
n	4	4	4
supplemental canavanine or canaline (g added kg^{-1} JB)	1–50	1–25	20

* measured by colorimetric examination of the pentacyanoammine Fe(III) complex. details of the method are presented in section 2.4.7.

which was five times greater than that used for the standards, did not produce any peaks that were attributable to either canavanine or canaline.

The chromatogram of extracted chick excreta (Fig. 3.2.2d) shows that there is an absence of canavanine and canaline peaks. Treatment of the excreta extract (10ul) with OPA, without prior treatment with acetonitrile precipitant, did not show any peaks with the same retention times as canavanine or canaline. Increasing the sensitivity five fold also, did not show any peaks for canavanine or canaline.

The concentration of canavanine in a sample of JB, estimated using a variety of methods, shows (Table 3.2.1) that there is fairly good agreement between the methods.

Surprisingly there was excellent agreement between the analysis of the H_2O extract from jack beans by HPLC and colorimetry. Lower values were obtained for the analysis of the hydrolysed samples by HPLC and IEC although results obtained using both techniques were closely correlated. Of the methods used, the colorimetric method was the most precise while the IEC method was least precise. It is interesting to note that hydrolysis of jack beans gives a reduction in canavanine content (ca. 60g kg^{-1} of canavanine) of a similar order to the reduction in mimosine content of *leucaena* which has been hydrolysed (Acamovic and D'Mello, 1981). Chromatography of hydrolysed soyabean meal did not produce any peaks with the same retention times as either canavanine or canaline. There also appears to be minimal interference in the colorimetric estimation of canavanine in the water extracts of jack beans.

Recoveries of supplemental canavanine and canaline from jack beans (Table 3.2.2) indicate that only small losses occurred during sample preparation and, that reproducibility of recovery was fairly good over the supplemental ranges studied.

Recovery of canavanine and canaline from chick excreta, as analysed by HPLC, was 1.002 (RSD =0.035; n=4) and 0.933 (RSD = 0.011; n=4) respectively. Additional canavanine and canaline were at levels between 1-50 and 1-25g kg^{-1} of excreta respectively. Reproducibility of recovery for both analytes was good but recovery of canaline was slightly low over the supplemental ranges.

Recovery of canaline added to chick serum (10-125nmol cm^{-3}) averaged 0.927 (RSD = 0.06; n=5) but addition of canavanine to the serum from a chick fed a canavanine free diet produced recoveries greater than 1.0 and RSD values were large at supplementary levels below 125nmol cm^{-3}. The recoveries became larger as supplementary canavanine decreased. It was subsequently found that the peaks

Table 3.3.1 Growth rate (GR) food intake (FI) and efficiency of food conversion (EFC) of chicks in experiment 1.

diet	GR ($g\ d^{-1}$)	FI ($g\ d^{-1}$)	EFC (weight gain/DM intake)
1.1	16.13	25.12	0.642
1.2	16.60	25.50	0.651
1.3	16.02	26.01	0.616
1.4	20.34	33.56	0.606
1.5	16.53	30.50	0.542
1.6	22.16	35.34	0.627
1.7	17.64	32.01	0.551
sem (18 df)	0.8425	1.1351	0.01837

Period of observation: 7 to 21 days of age.

which surround the canavanine peak interfered with canavanine when the Spherisorb column was used for the analysis. Interference of the canavanine peak in serum was overcome by using the Hypersil column which gave a mean recovery of 1.011 (RSD = 0.036; n=5) at supplementary levels between 10 and 150nmol cm^{-3} of serum. The Hypersil column was used for subsequent analysis of canavanine in serum from chicks and in JB. The results for the content in the various samples are presented in chapter 2 and in the remainder of this chapter.

3.3 Chick experiments.

3.3.1 Experiment 1.

Two birds in this experiment died. One of these birds was fed the basal LLM diet (diet 1.1) while the other was allocated to the basal diet supplemented with PEG4000 (diet 1.3). The other birds remained healthy and active throughout the experimental period of 14d. Growth and efficiency of food conversion (EFC; liveweight gain/ DM intake) from 7 to 21 days of age are shown in Table 3.3.1. The growth rate of chicks fed the starter diet during the experimental period was 24.63g $chick^{-1}$ d^{-1}. These chicks were not included in the design of the experiment but were used to calculate the RGR values of the chicks in this experiment.

LLM diets supplemented with PEG4000 alone (diets 1.2 & 1.3) did not produce growth rates which were significantly different ($P>0.05$) from the basal LLM (diet 1.1) diet. Supplementation of the basal LLM diet with $Fe_2(SO_4)_3$ (12g kg^{-1}; diet 1.4) produced a significant ($P<0.05$) increase in growth over the basal diet. Inclusion of PEG4000 as an added supplement to Fe(III) (diet 1.6) produced growth rates which were significantly better ($P<0.01$) than the LLM basal diet. The superior growth of birds on the Fe(III) and PEG4000 supplemented diet (diet 1.6), compared to the diet with Fe(III) supplementation alone (diet 1.4), was not significant ($P>0.05$). Supplementation of the LLM diet with $Fe_2(SO_4)_3$ at 24g kg^{-1} (diets 1.5 & 1.7) did not significantly ($P>0.05$) affect bird growth with respect to the LLM basal diet and these were significantly lower($P<0.05$) than birds fed diets supplemented with a lower quantity of Fe(III) (diets 1.4 & 1.6).

A notable effect of inclusion of Fe(III) into LLM diets was that food intake increased significantly ($P<0.001$) for chicks fed these diets compared with the LLM basal diet (diet 1.1). Inclusion of PEG4000 alone (diets 1.2 & 1.3) caused a slight but insignificant ($P>0.05$) increase in food intake compared to birds fed the LLM basal diet.

Table 3.3.2 Carcass composition of chicks at 21d of age in experiment 1.

diet	gross energy (MJ kg^{-1} DM)	fat (g kg^{-1} DM)	nitrogen (g kg^{-1} DM)
1.1	23.56	179.2	99.87
1.2	24.02	193.0	99.31
1.3	23.99	195.9	100.54
1.4	24.05	221.4	92.87
1.5	23.91	204.4	97.92
1.6	23.63	191.2	98.47
1.7	23.62	178.3	101.39
sem (18 df)	0.3297	13.0383	1.7962

Table 3.3.3 Efficiency of deposition of nitrogen (END) and energy (EDE) during the 14d experimental period in chicks in experiment 1.

diet	END	EDE	EDE*
1.1	0.472	0.210	0.210
1.2	0.448	0.224	0.230
1.3	0.475	0.217	0.231
1.4	0.435	0.220	0.220
1.5	0.437	0.198	0.198
1.6	0.486	0.215	0.222
1.7	0.427	0.184	0.196
sem (18 df)	0.01466	0.00961	0.00982

* energy intake corrected for energy in PEG4000 = 26.38MJ kg^{-1}.

Inclusion of $Fe_2(SO_4)_3$ at 24g kg^{-1} (diets 1.5 & 1.7) caused a significant ($P<0.01$) decrease in EFC (Table 3.3.1) compared with that for birds fed the LLM basal diet (diet 1.1). The EFC of chicks fed the other diets were not significantly different ($P>0.05$) from chicks fed the basal diet (diet 1.1).

From the carcass composition data (Table 3.3.2) it is apparent that carcass energy content does not significantly differ ($P>0.05$) between chicks fed the various diets.

The fat content of the carcasses from birds fed the PEG4000 free diet with the low level of Fe(III) (diet 1.4) is significantly higher ($P<0.05$) while caracasses from birds fed the other diets are not appreciably ($P>0.05$) different, from the those fed the basal diet. The higher carcass fat content of birds fed diet 1.4 is reflected in the lower ($P<0.05$) carcass nitrogen content of these birds compared to those from the basal diet. The carcass nitrogen content of birds fed the other diets are essentially the same ($P>0.05$) as those fed the basal diet. The nitrogen content of animals from the diets with additional Fe(III) only, (diets 1.4 & 1.5) are not significantly different ($P>0.05$) from each other but the diet with the Fe(SO4)3 added at 12g kg^{-1} (diet 1.4) produced animals with a lower ($P< 0.05$) nitrogen content than the remaining diets.

Efficiency of nitrogen deposition (END= N deposited in carcass/N intake over the experimental period) is lower ($P<0.05$) for birds fed the diet containing the highest amount of Fe(III) and PEG4000 (diet 1.7) than those fed the basal diet (Table 3.3.3).

The composition of the other diets did not affect ($P>0.05$) the END compared with chicks fed the basal diet (1.1). The diet containing the highest combined quantity of Fe(III) and PEG4000 (diet 1.7) reduced ($P<0.05$) END compared with that for birds fed the diet containing Fe(III) and PEG4000 at the lower level of inclusion (diet 1.6). It is also notable that the diet with highest levels of supplementary PEG4000 and Fe(III) (diet 1.7) produced a significantly lower END than the diet containing the same quantity of PEG4000 but no additional Fe(III) (diet 1.3).

The EDE in chicks fed any of the diets is not significantly different from that in the birds fed the basal diet even when the energy intake has been corrected for the energy in PEG4000. The uncorrected EDE is significantly lower ($P<0.05$) for the diet supplemented with largest amounts of Fe(III) and PEG4000 (diet 1.7), compared to the diet with the lowest supplemental Fe(III) and no PEG4000

Table 3.3.4 Mean daily mimosine and 3-hydroxy-4(1H)-pyridone (3,4-DHP) intake (mg kg^{-1} liveweight) determined during the 14d experimental period.

diet	mimosine intake	3,4-DHP intake
1.1	277.8	55.55
1.2	277.0	55.40
1.3	290.0	58.00
1.4	313.5	62.70
1.5	346.0	69.20
1.6	307.8	61.55
1.7	332.0	66.40
sem (18 df)	9.3598	1.8720

Table 3.3.5 Relationship of mimosine and 3-hydroxy-4(1H)-pyridone (3,4-DHP) intake to output in chicks fed Fe(III) supplemented or unsupplemented Leucaena leaf meal diets.

diet	mimosine (out/in)	3,4-DHP (out/in)	mimosine + 3,4-DHP (total mols; out/in)
1.1	0.866(0.0180)	0.966(0.0556)	0.896(0.0224)
1.2	nd	nd	nd
1.3	nd	nd	nd
1.4	1.099(0.0180)	1.027(0.0298)	1.076(0.0204)
1.5	0.905(0.0571)	0.989(0.1267)	0.931(0.0707)
1.6	1.054(0.0540)	0.995(0.1300)	1.035(0.1457)
1.7	0.891(0.0933)	1.592(0.2098)	1.112(0.1236)

nd: not determined.
period of observation: 16 to 18 days of age.
values in brackets are sem for each mean (n=4).

(diet 1.4). The differences in EDE are in agreement with the fat contents (Table 3.3.2) of the birds fed these diets (1.4 & 1.7).

Daily mimosine and 3,4-DHP intakes related to liveweight of chicks (calculated as the intake of mimosine and 3,4-DHP over the 14 day experimental period divided by the final liveweight) clearly shows (Table 3.3.4) that supplementation of the LLM diets with Fe(III) (diets 1.4-1.7) allows a significant ($P<0.05$) increase in the intake of mimosine and 3,4-DHP when related to liveweight. The highest levels of supplementation of Fe(III) (diets 1.5 & 1.7) produced the highest ($P<0.001$) mimosine and 3,4-DHP intakes when related to body weight.

The ratio of mimosine and 3,4-DHP intake to that excreted is shown in table 3.3.5. Most of the ingested mimosine in the basal diet (1.1) was excreted although the ratio was significantly lower ($P<0.05$) than unity. The combined molar recovery of mimosine and 3,4-DHP in the excreta of chicks fed the LLM basal diet (diet 1.1) was significantly less than unity ($P<0.05$). The proportion of mimosine excreted by chicks fed the Fe(III) supplemented diets are not significantly different from unity ($P>0.05$) and higher than for birds fed the LLM basal diet (diet 1.1). It is notable that the lower recovery of mimosine is accompanied by an increased recovery of 3,4-DHP for the diet containing the highest supplement of Fe(III) and PEG4000 (diet 1.7). Only diet 1.7 produced a significantly higher ($P<0.05$) combined molar excretion of mimosine and 3,4-DHP than birds fed the basal diet. The combined molar excretion of mimosine and 3,4-DHP for chicks fed all diets (except diet 1.1) was not significantly different ($P>0.05$) from unity.

3.3.2 Experiment 2.

Two birds died during the 14d experimental period and both were fed the LLM diet with $Fe_2(SO_4)_3$ supplementation at 1.6g kg^{-1} (diet 2.5). Post mortem examination did not associate the diet with the cause of death. Other birds remained active and healthy throughout the 14d experimental period.

There was a highly significant ($P<0.001$) depression in growth rate (Table 3.3.6) when birds were fed an unsupplemented LLM diet (diet 2.4) compared with a control soya bean meal diet (diet 2.1).

Supplementation of the control diet (2.1) with Fe(III) (diets 2.2 & 2.3) did not significantly alter ($P>0.05$) growth rate, food intake or EFC. Addition of Fe(III) to the LLM basal diet (diet 2.5) significantly increased ($P<0.05$) growth rate with

Table 3.3.6 Growth rate (GR), food intake (FI) and efficiency of food conversion (EFC) of chicks in experiment 2.

diet No.	GR (g chick^{-1} d^{-1})	FI	EFC (weight gain/DM intake)
2.1	26.71	40.90	0.653
2.2	24.94	39.71	0.628
2.3	25.74	40.16	0.641
2.4	17.97	27.69	0.649
2.5	21.06	32.30	0.652
2.6	19.36	32.48	0.596
2.7	21.53	35.30	0.610
2.8	22.82	36.05	0.633
sem (21 df)	0.78428	1.0777	0.01559

period of observation: 7 to 21 days of age.

Table 3.3.7 Carcass composition of 21d old chicks in experiment 2.

diet	gross energy (MJ kg^{-1} DM)	fat (g kg^{-1} DM)	nitrogen (g kg^{-1} DM)
2.1	25.36	274.6	88.31
2.2	24.88	261.5	90.36
2.3	24.82	251.8	90.94
2.4	23.62	200.2	94.20
2.5	23.71	220.1	90.58
2.6	23.70	209.7	93.55
2.7	23.72	212.1	94.49
2.8	24.27	246.1	88.16
sem (21 df)	0.2982	15.1321	1.7790

the exception of birds fed the diet supplemented with the lowest quantity of Fe(III) and supplemented with PEG4000 (diet 2.6). All chicks fed an LLM diet grew more slowly ($P<0.05$) than birds fed the control diet (2.1). Addition of PEG4000 did not ($P>0.05$) affect growth, nor did the variation in supplemental Fe(III) have any effect on growth of chicks fed LLM and Fe(III) supplemented diets (diets 2.5, 2.6, 2.7 & 2.8). Only birds fed the LLM diet with the lowest supplemental Fe(III) and PEG4000 (diet 2.6) induced a significantly lower ($P<0.05$) EFC than control fed birds.

Chicks fed the LLM basal diet and those supplemented with Fe(SO4)3 at 1.6g kg^{-1} (diets 2.4, 2.5 & 2.6) had much reduced food intake ($P<0.001$) compared to chicks fed the control diet (diet 2.1). Those chicks fed the LLM diets with 4.8g kg^{-1} of additional $Fe_2(SO_4)_3$ (diets 2.7, 2.8) had higher food intakes than than those supplemented with the lower levels of Fe(III) but they were still lower than birds fed the control diet. Addition of Fe(III) to the LLM diets allowed a significant increase in food intake of birds fed these diets compared with chicks fed the LLM basal diet. Compared to chicks fed the LLM basal diet, food intakes were higher ($P<0.001$) for chicks fed the diets containing more Fe(III) (diets 2.7 & 2.8) than those ($P<0.01$) fed diets containing the lower amounts of Fe(III) (diets 2.5 & 2.6).

The data for carcass composition (Table 3.3.7) show that birds fed diets containing LLM had significantly lower ($P<0.05$) energy and fat contents, with the exception of birds fed the diet supplemented with PEG4000 and the highest Fe(III) (diet 2.8), than birds fed soya bean diets (diets 2.1, 2.2 & 2.3). Addition of Fe(III) to the diets had no effect ($P>0.05$) on either energy or fat content of the carcasses. Similarly, PEG4000 had no effect on the fat and energy components of the birds fed the LLM based diets (diets 2.6 & 2.8). The nitrogen content of the carcasses were significantly higher ($P<0.05$) for birds fed the LLM basal diet (diets 2.4) and the LLM diets supplemented with PEG4000 and the lowest level of Fe(III), and the highest level of Fe(III) alone (diets 2.6 & 2.7 respectively), compared with the control fed birds (diet 2.1). Birds fed the diet containing PEG4000 and the highest amount of Fe(III) (diet 2.8) had a lower ($P<0.05$) nitrogen content than the birds fed the basal LLM diet (diet 2.4).

Supplementation of the soya bean control diet with Fe(III) induced an increase in END compared with control fed chicks (Table 3.3.8) but this was only significant ($P<0.05$) when $Fe_2(SO_4)_3$ was added at 12g kg^{-1} diet (2.3). The LLM diets supplemented with the lower amount of Fe(III) (diets 2.5 & 2.6) showed a significantly lower ($P<0.05$) END than the soya bean control fed birds. Of these diets the one without PEG4000 supplementation (diet 2.5) produced a

Table 3.3.8 Efficiency of deposition of nitrogen (END) and energy (EDE) in 21d old chicks in experiment 2.

diet No.	END	EDE
2.1	0.476	0.300
2.2	0.516	0.282
2.3	0.545	0.281
2.4	0.463	0.222
2.5	0.414	0.207
2.6	0.423	0.213
2.7	0.481	0.216
2.8	0.467	0.241
sem (21 df)	0.014064	0.008151

Table 3.3.9 Mean daily mimosine and 3-hydroxy-4(1H)-pyridone (3,4-DHP) intake (mg kg^{-1} liveweight) during the 14 day experimental period.

diet No.	mimosine intake	3,4-DHP intake
2.1	0	0
2.2	0	0
2.3	0	0
2.4	270.5	54.10
2.5	289.3	57.86
2.6	300.9	60.18
2.7	306.3	61.25
2.8	296.9	59.38
sem (12 df)	7.1110	1.4222

Table 3.3.10 Relationship of mimosine and 3-hydroxy-4(1H)-pyridone (3,4-DHP) intake to output in chicks fed supplemented or unsupplemented Leucaena leaf meal diets.

diet No.	mimosine (out/in)	3,4-DHP (out/in)	mimosine + 3,4-DHP (total mols; out/in)
2.1	-----	-----	-----
2.2	-----	-----	-----
2.3	-----	-----	-----
2.4	0.992(0.0213)	0.805(0.0220)	0.931(0.0064)
2.5	0.950(0.0405)	0.849(0.0344)	0.919(0.0171)
2.6	1.035(0.0538)	0.749(0.0790)	0.945(0.0604)
2.7	0.990(0.0149)	0.892(0.0204)	0.959(0.0071)
2.8	1.039(0.0218)	0.828(0.0402)	0.972(0.0093)

period of observation: 19-21 days of age.
values in brackets are sem for each mean (n=4)

significantly reduced (P<0.05) END when compared with chicks fed the LLM basal diet (2.4).

The EDE (Table 3.3.8) is highest (P<0.05) for chicks fed the soya/maize diets (diets 2.1-2.3) Addition of Fe(III) to the soya/maize diets (diets2.2, 2.3) caused a slight decrease in EDE compared to the control diet (2.1) but this was not significant. All chicks fed the LLM diets had a lower (P<0.05) EDE than birds fed the control diet. Addition of PEG4000 and Fe(III) to the LLM diets had no effect on EDE except for inclusion of these at the highest level (diet 2.8) which caused a significant increase (P<0.05) compared to animals fed the LLM basal diet (2.4).

The mean daily intakes of mimosine and 3,4-DHP relative to liveweight, for birds fed the LLM diets are presented in table 3.3.9. Addition of Fe(III) and PEG4000 to these diets show that daily mimosine and 3,4-DHP intakes are significantly higher (P<0.05) for the diets supplemented with the low level of Fe(III) and PEG4000 and those with the higher level of Fe(III) (diets 2.6, 2.7 & 2.8). The diet supplemented with the low level of Fe(III) only, (diet 2.5) caused a slightly higher intake of mimosine and 3,4-DHP than birds fed the basal LLM diet but the increase was not significant (P>0.05).

Irrespective of whether the LLM diets were supplemented or not, all the mimosine ingested was excreted (P>0.05) by the chicks (Table 3.3.10). The supplementation of the LLM diets with PEG4000 and Fe(III) did not significantly (P>0.05) alter the excretion of mimosine compared to LLM basal (2.4) fed birds. Although the ingested mimosine could be accounted for in the excreta, 3,4-DHP recoveries were low (Table 3.3.10) and not significantly different (P>0.05) from birds fed the basal LLM diet (2.4). When the combined molar output to input of mimosine and 3,4-DHP are related they are not significantly (P>0.05) different from each other and only birds fed the diet (2.5) supplemented with least (Fe(III) only, differed (P<0.05) from unity.

3.3.3 Experiment 3.

All birds in this experiment survived the experimental period of 12 days. Birds fed diets containing the *leucaena* seeds (diets 3.3, 3.4 & 3.5) had a greater tendency to sit than did birds fed the other diets however they were otherwise healthy. The excreta from birds fed the diets containing LS without added Fe(III) (diets 3.3 & 3.5), had a pronounced red/orange colour. The cause of the colouration is unknown but may be due to the formation of the Fe(III)/phosphate complexes with mimosine and 3,4-DHP which have been observed in mouse

Table 3.3.11 Growth rate (GR), food intake (FI), efficiency of food conversion (EFC), efficiency of nitrogen retention (ENR) of chicks and apparent metabolisable energy (AME) of diets used in experiment 3.

	diet No.					sem
	3.1	3.2	3.3	3.4	3.5	(12df)
GR (g chick^{-1} d^{-1})	26.49	16.18	20.40	23.25	14.59	0.5132
FI (g chick^{-1} d^{-1})	37.47	24.97	31.78	37.08	24.73	0.6686
EFC	0.707	0.648	0.642	0.627	0.590	0.01047
ENR	0.589	0.557	0.590	0.633	0.560	0.01608
AMEc (MJ kg^{-1} DM)	13.14	13.20	13.04	13.00	12.93	0.1428
AMEn (MJ kg^{-1} DM)	12.38	12.46	12.27	12.19	12.21	0.1307

GR, FI, EFC values were obtained during the 12d experimental period.
period of observation for AME: 17-19days of age.

Table 3.3.12 Daily mimosine intake and mimosine and 3-hydroxy-4(1H) -pyridone (3,4-DHP) excreted:ingested ratios.

diet No.	mimosine intake (mg kg^{-1} liveweight)	mimosine (out/in)	mimosine + 3,4-DHP (total mols; out/in)
3.1	0	-----	-----
3.2	303.3	0.698(0.0509)	0.689(0.0309)
3.3	329.7	0.744(0.0306)	0.923(0.0267)
3.4	350.5	0.733(0.0324)	0.908(0.0514)
3.5	467.8	0.739(0.0245)	0.899(0.0391)
sem (9 df)	4.5486	----	----

period of observation: 7-19 days of age for mimosine intake and 17-19 days of age for output:intake ratio.
values in brackets: sem for each mean (n=4).

diets (Hegarty, Lee, Christie, Court and Haydock, 1979) and which are reported to be less readily absorbed from the gastrointestinal tract than mimosine and 3,4-DHP (Tsai and Ling, 1974). The diets containing LS with or without Fe(III) did not show signs of the colouration.

The experiment had to be terminated after 12 days because there was insufficient diet available to continue to 14 days.

Birds fed the control soya bean meal and maize diet (diet 3.1) grew at a significantly faster rate ($P<0.001$) than chicks fed any of the other diets (Table 3.3.11). Birds fed the unsupplemented LLM diet (diet 3.2) had a highly significant reduced ($P<0.001$) growth rate than did birds fed the two diets containing LS at 65g kg^{-1} (diets 3.3 & 3.4). Animals fed the diet (diet 3.5) containing the higher concentration of LS (95g kg^{-1}) grew at a significantly poorer rate ($P<0.05$) than birds fed the LLM basal diet (diet 3.2). Incorporation of Fe(III) into the LS diet (diet 3.4) caused a significant increase ($P<0.01$) in growth rate compared with chicks fed the similar diet without additional Fe(III) (diet 3.3).

As in previous experiments inclusion of LLM (diet 3.2) depressed food intake significantly ($P<0.001$) compared with control fed birds (diet 3.1). Inclusion of LS at 95g kg^{-1} of diet (diet 3.4) caused a similar reduction, while inclusion of LS at 65g kg^{-1} (diet 3.3) did not cause such a severe depression in food intake although both were significantly lower ($P<0.001$) than for control fed birds. Food intake for chicks fed the LS diet supplemented with Fe(III) (diet 3.4) was not significantly lower than for control fed birds. Chicks fed the diets with 65g kg^{-1} LS (diets 3.3, 3.4) had significantly higher ($P<0.001$) food intakes than the LLM fed birds.

Dietary composition had a profound effect on EFC (Table 3.3.11). The EFC was poorest ($P<0.001$) for birds fed diets containing LS but was also significantly reduced ($P<0.01$) for LLM fed birds (diet 3.2), when compared with control birds. The birds fed the LLM diet (diets 3.2) and those fed the LS supplemented diets (diets 3.3 & 3.4) with or without added Fe(III), did not differ significantly ($P>0.05$) in their ability to utilise the diets. There was, however a significant reduction ($P<0.01$) in the EFC of birds fed the diet (diets 3.5) supplemented with the largest amount of LS.

Inclusion of either LLM or LS had no effect ($P>0.05$) on ENR (Table 3.3.11) of chicks fed these diets compared with those fed the control diet (diet 3.1)

Inclusion of Fe(III) into the diet containing LS (diet 3.4) did, however elicit a higher ($P<0.05$) ENR than birds fed the diet (diet 3.2) containing LLM.

The AME(c) values (Table 3.3.11) of the diets were determined to be the same ($P>0.05$) while those for AME(n) also did not significantly ($P>0.05$) differ from each other.

Daily mimosine intake, related to liveweight, and the ratio of excreted mimosine and 3,4-DHP to intake are presented in table 3.3.12. In this experiment 3,4-DHP was not detectable in the LS and therefore no intake data for 3,4-DHP is available for diets containing LS.

Chicks fed diets containing LS had higher mimosine intakes relative to liveweight (Table 3.3.12) than birds fed the diet containing LLM (diet 3.2). Chicks fed the unsupplemented diet containing the low level of LS (3.3) had a significantly higher ($P<0.01$) mimosine intake and grew at a faster rate (Table 3.3.11) than birds fed the LLM based diet. Mimosine intakes were considerably higher ($P<0.001$) for the birds fed the Fe(III) supplemented LS diet (diet 3.4) and the diet containing the highest level of LS (diet 3.5) than for those fed the LLM based diet (diet 3.2).

The ratio of mimosine excreted to that ingested (Table 3.3.12) was slightly higher for chicks fed diets containing LS than those fed LLM diets, however the differences were not significant ($P>0.05$). The LLM fed birds did excrete less ($P<0.001$) mimosine + 3,4-DHP, relative to that ingested, than birds fed the LS diets. In this experiment Fe(III) supplementation of the LS diet (diet 3.4) did not enhance mimosine excretion. Mimosine output relative to intake was significantly less ($P<0.05$) than unity for chicks fed all the diets in this experiment. Only chicks fed the LLM diet (diet 3.2) had a ratio of excretion of mimosine and 3,4-DHP to intake, which was significantly lower ($P<0.05$) than unity.

3.3.4 Experiment 4.

There was no mortality amongst birds in this experiment, all animals remaining healthy throughout the 14 day experimental period.

In common with previous experiments, chicks fed the control diet (diet 4.1) grew at a much faster rate (Table 3.3.13) than birds fed LLM diets; this difference being highly significant ($P<0.001$).

Table 3.3.13 Daily growth rate (GR), food intake (FI), and efficiency of food conversion (EFC) of chicks on experiment 4.

diet No.	GR (g chick^{-1} d^{-1})	FI (g chick^{-1} d^{-1})	EFC (weight gain/ DM intake)
4.1	29.16	48.03	0.607
4.2	21.78	35.65	0.611
4.3	23.32	40.49	0.596
4.4	22.70	35.64	0.627
sem (6 df)	0.5757	1.0960	0.00595

period of observation: 7 to 21d of age.

Table 3.3.14 Mean daily mimosine and 3-hydroxy-4(1H)-pyridone (3,4-DHP) intakes (mg kg^{-1} liveweight) and output : intake data.

	diet No. 4.1	4.2	4.3	4.4	sem (6 df)
mimosine intake (mg kg^{-1})	0	284.7	297.2	280.2	3.2767
3,4-DHP intake (mg kg^{-1})	0	56.95	59.44	56.05	0.6553
mimosine (out/in)	-----	0.767	0.779	nd	-----
sem (n=4)	-----	0.0190	0.0709	----	-----
3,4-DHP (out/in)	-----	0.675	0.836	nd	-----
sem (n=4)	-----	0.0096	0.0700	----	-----
mimosine + 3,4-DHP (out/in)	-----	0.738	0.797	nd	-----
sem (n=4)	-----	0.0148	0.0655	----	-----

period of observation: mimosine and 3,4-DHP intakes: 7-21 days of age.
period of observation for output:intake ratios: 19-21 days of age.

Table 3.3.15 Daily growth rate (GR), food intake (FI) and efficiency of food conversion (EFC) of chicks in experiment 5.

diet No.	GR (g chick^{-1} d^{-1})	FI (g chick^{-1} d^{-1})	EFC (weight gain/DM intake)
5.1	29.07	41.95	0.693
5.2	30.57	44.30	0.690
5.3	17.31	27.82	0.622
5.4	28.18	41.62	0.677
5.5	25.51	40.43	0.631
5.6	19.99	30.99	0.645
5.7	28.63	42.16	0.679
5.8	20.61	33.30	0.619
sem (21 df)	0.85948	1.0638	0.012016

period of observation: 7-20 days of age.

The growth rate of birds fed the Fe(II) supplemented or unsupplemented LLM diets were not significantly different ($P>0.05$) from each other although birds fed the basal diet (diet 4.2) grew slowest.

Food intake of LLM fed birds was significantly lower ($P<0.001$) than for birds fed the control diet (diet 4.1). There was no significant difference ($P>0.05$) in food intake for chicks fed the Fe(II) supplemented diets (diets 4.3 & 4.4 compared to those fed the LLM basal diet (diet 4.2).

There was no difference ($P>0.05$) between the EFC of chicks fed the control diet (diet 4.1) and of those fed the other diets. The EFC of the animals fed the Fe(II) supplemented LLM diets (diets 4.3 & 4.4) compared with those fed the LLM basal diet (diet 4.2) were not significantly ($P>0.05$) different. Birds which consumed the LLM diet with highest Fe(II) supplementation (diet 4.4) had a higher ($P<0.05$) EFC than those given the diet supplemented with 1.55g FeSO4 (diet 4.3).

Supplementation of the basal LLM diet (diet 4.2) with the low level of Fe(II) (diet 4.3) significantly increased ($P<0.05$) the intake of mimosine and 3,4-DHP (Table 3.3.14). Mimosine excretion was not enhanced ($P>0.05$) by the inclusion of additional Fe(II) in the LLM diet (diet 4.3). Relative excretion of mimosine, 3,4-DHP and mimosine + 3,4-DHP, were significantly lower ($P<0.05$) than unity for chicks fed the LLM basal diet (diet 4.2) while chicks fed the supplemented diet (diet 4.3) did not have values significantly less ($P>0.05$) than unity.

3.3.5 Experiment 5.

The experimental period was limited to 13 days due to lack of diet. During the experimental period there was one mortality while other chicks remained healthy and active. Excreta from birds fed the diet (5.8) containing supplementary mimosine, had a pronounced red/orange colour similar to excreta from the animals fed LS in experiment 4.

There was a slight, but not significant ($P>0.05$), increase in growth rate (Table 3.3.15) when birds were fed the soya bean diet (diet 5.2) with supplemental PVP-40, when compared to that for chicks fed the control diet (5.1). As with previous experiments, the basal LLM diet (diet 5.3) caused a severe depression in growth and food intake ($P<0.001$) relative to control fed birds. Supplemental Fe(III), PEG4000 and cholesterol (diet 5.4) dramatically improved growth rate, food intake ($P<0.001$) and EFC ($P<0.01$) compared to animals fed the LLM basal diet (diet 5.3). Growth rate and food intake of birds fed this diet (diet 5.4) were

slightly lower than those fed the control diet but the difference was not significant (P>0.05).

Inclusion of PVP-40 in the LLM diet (diet 5.5) rather than PEG4000 improved growth rate and food intake above birds fed the basal LLM diet (diet 5.3); this improvement was also highly significant (P<0.001) although growth rate but not food intake was significantly lower P<0.05) than birds fed the equivalent diet (diet 5.4) with PEG4000. Cooking LLM (diet 5.6) significantly improved (P<0.05) the growth of chicks compared to those fed the LLM basal diet. Addition of Fe(III), PEG4000 and cholesterol to the cooked LLM diet (5.7) had a highly significant (P<0.001) effect on growth compared to birds fed the basal LLM diet (5.3) and those fed the unsupplemented diet containing cooked LLM (5.6). Addition of mimosine to a soya/maize diet (5.8) caused a significant (P<0.001) and severe growth depression compared to control birds. The growth of birds fed supplemental mimosine (diet 5.8) was, however, significantly higher (P<0.05) than birds fed the LLM basal diet.

Food intake for chicks fed the dits containing autoclaved LLM, and supplemental mimosine (diets 5.6, 5.8) were significantly lower than for control fed birds. Significant increases in food intake were obtained for chicks fed the autoclaved LLM (P<0.05), mimosine (P<0.01) and the other treated LLM diets (P<0.001).

There was no significant difference (P>0.05) in EFC (Table 3.3.15) between birds fed the control diet (5.1) and those fed the soya/maize diet (5.2) with supplemental PVP-40. Animals fed the LLM basal diet (5.3) had a reduced EFC compared to control fed birds; the reduction being highly significant (P<0.001). Chicks fed the LLM diet (5.5) supplemented with PVP-40 had a slightly better EFC than birds fed the basal LLM diet but the improvement observed was not significant (P>0.05). Cooking LLM caused a slight improvement in the EFC of birds fed this diet (5.6) compared to those fed the basal LLM diet (5.3) but the improvement was not significant (P>0.05). Addition of Fe(III), PEG4000 and cholesterol to the cooked LLM (diet 5.7) caused an improvement in EFC and, although the EFC was less than birds fed the control diet (5.1), the difference was not significant. The EFC for birds fed the cooked LLM and supplemented diet (5.7) however, was significantly higher (P>0.01) than for the birds fed the LLM basal diet (5.3). The EFC of animals fed the mimosine supplemented diet (5.8) was slightly lower, but not significantly (P>0.05) so, than birds fed the basal LLM diet. There was a highly significant difference (P<0.001) between the EFCs of birds fed the control diet and those fed the mimosine supplemented diet.

Table 3.3.16 Carcass composition of chicks in experiment 5.

diet No.	ash	nitrogen	fat	energy
	(--------g kg^{-1} DM---------)			(MJ kg^{-1} DM)
5.1	90.02	90.27	278.5	35.54
5.2	nd	nd	nd	nd
5.3	94.11	95.44	199.0	24.21
5.4	89.88	91.74	181.5	24.73
5.5	97.17	94.45	188.3	24.42
5.6	94.33	89.83	262.7	25.01
5.7	94.00	91.85	188.2	24.62
5.8	92.32	90.55	283.6	25.5
sem (18 df)	2.0964	1.5341	14.165	0.2324

chicks at 20d of age were used for carcass composition studies.
nd: not determined.

Table 3.3.17 Efficiency of nitrogen retention (ENR), classical and N corrected apparent metabolisable energy (AME) for diets in experiment 5.

diet No.	ENR	AME(c)	AME(n)
		(----MJ kg^{-1} DM------)	
5.1	0.564	13.83	13.06
5.2	0.539	13.89	13.14
5.3	0.515	13.41	12.72
5.4	0.535	13.00	12.30
5.5	0.508	12.45	11.78
5.6	0.560	13.89	13.09
5.7	0.560	12.85	12.07
5.8	0.539	13.92	13.17
sem (21 df)	0.01751	0.15993	0.14467

period of excreta collection: 16-20 days of age.

The ash content of the carcasses (Table 3.3.16) was highest for chicks fed the diet supplemented with Fe(III), PVP-40 and cholesterol (diet 5.5); it was significantly higher than for birds fed the control diet (diet 5.1).

The nitrogen content of birds fed the LLM basal diet (diet 5.3) was significantly higher ($P<0.05$) than those fed the control diet (Table 3.3.16). The nitrogen content of animals fed the control diet, however, was not significantly different ($P>0.05$) from those of birds fed the other diets. Chicks fed the diet containing the cooked LLM (diet 5.6), but otherwise unsupplemented, and that with added mimosine (diet 5.8) caused a significant ($P<0.05$) reduction in the carcass nitrogen content compared with chicks fed the LLM basal diet (diet 5.3).

Dietary composition had a considerable effect on the carcass fat content (Table 3.3.16). The control diet (diet 5.1), the diet supplemented with cooked LLM (diet 5.6) and the mimosine supplemented (diet 5.8) produced carcasses which were significantly ($P<0.05$) fatter than birds fed the other diets. It is interesting to note that birds fed the diets (diet 5.1 & 5.8) with lowest supplemental oil, had the highest fat content. Only the diet supplemented with cooked LLM (diet 5.6) yielded carcasses with a significantly higher fat content than birds fed the LLM basal diet (diet 5.3) which had an equal supplement of oil.

Carcass energy is much reduced ($P<0.001$) in birds fed the control diet (diet 5.1) compared with those fed the LLM basal diet (diet 5.3). The diets containing cooked LLM (diet 5.6) and added mimosine (diet 5.8) produced birds with higher ($P<0.01$) components of energy than birds fed the basal LLM diet (diet 5.3).

Only chicks fed the LLM diet (diet 5.5) with PVP-40 had a significantly lower ($P<0.05$) ENR (Table 3.3.17) than chicks fed the control diet (diet 5.1). The ENR for other diets were not significantly ($P>0.05$) different from the control diet. Correction of the ENR of diet 5.5 for the nitrogen content of PVP-40 (125g kg^{-1}), which is unavailable to the chick, removed the difference between ENR from birds on this diet and those fed the control diet.

The AME(c) and AME(n) values (Table 3.3.17) for the LLM diets (diets 5.4, 5,5 & 5.7) with supplements of cholesterol, Fe(III) and PEG4000 or PVP-40 and highest additional vegetable oil (Table 2.13.6) were lower ($P<0.001$) than those for the LLM basal diet (diet 5.3). Diets 5.5 and 5.7 had similarly lower ($P<0.001$) AME values than the control diet. There was a smaller difference between the AME of the control diet and that of the LLM diet supplemented with PEG4000,

Table 3.3.18 Efficiency of nitrogen deposition (END) and efficiency of deposition of energy (EDE) over the 13d experimental period.

diet No.	END	EDE
5.1	0.472	0.290
5.2	nd	nd
5.3	0.411	0.194
5.4	0.471	0.237
5.5	0.454	0.218
5.6	0.428	0.244
5.7	0.454	0.238
5.8	0.431	0.263
sem (18 df)	0.01475	0.00904

nd: not determined.

Table 3.3.19 Mean daily mimosine and 3-hydroxy-4(1H)-pyridone (3,4-DHP) intake (mg kg^{-1} liveweight), serum alanine amino transferase (ALT) and aspartate amino transferase (AST) activity.

diet No.	mimosine (intake)	3,4-DHP (intake)	ALT *(units dm^{-3} at 37°C)	AST *(units dm^{-3} at 37°C)
5.1	0.000	0.00	5.13	149.1
5.2	nd	nd	5.55	154.9
5.3	316.3	63.25	5.88	159.3
5.4	308.9	61.77	4.38	140.1
5.5	324.0	64.80	6.00	157.3
5.6	296.9	59.38	6.50	171.8
5.7	309.8	61.96	5.58	162.9
5.8	326.3	0.00	5.75	134.0
sem (15 df)	8.0155 (12 df)	1.7389 sem (21 df)	0.5830	8.4228

serum samples obtained from chicks at 20 days old.
* An activity of 1 unit dm^{-3} is defined as a change in absorbance of 0.001 absorbance units min^{-1} at 340nm using the procedure described (sect 2.11 & 2.12).

cholesterol and Fe(III) (diet 5.4) but the discrepancy still remained significant ($P<0.01$).

Chicks fed the control diet (diet 5.1) had a significantly higher ($P<0.01$) END (Table 3.3.18) than birds fed the basal LLM diet (diet 5.3). Other END values did not differ significantly ($P>0.05$) from that for the control diet. The LLM diet supplemented with PEG4000, Fe(III) and cholesterol (diet 5.4) caused a significant increase ($P<0.05$) in the END compared with the LLM basal diet. Other END values were not significantly different ($P>0.05$) from the LLM basal diet.

All LLM diets produced significantly lower ($P<0.05$) EDE values than the control diet (Table 3.3.18) irrespective of the fact that the LLM diets had the highest additional oil contents (Table 2.13.6). The LLM diet with supplemental PVP-40 (diet 5.4) was the only diet containing LLM to have an EDE value which was not significantly higher ($P>0.05$) than that for the LLM basal diet. The diet containing added mimosine (diet 5.8) induced an EDE which was much larger ($P<0.001$) than that for chicks fed the LLM basal diet.

Although there is some variation in mean daily mimosine and 3,4-DHP intakes (Table 3.3.19) between birds fed the different diets, none differ significantly ($P>0.05$) from birds fed the basal LLM diet.

The serum ALT levels (Table 3.3.19) for birds fed the different diets varied slightly however the levels were not significantly ($P>0.05$) different from birds fed the control or basal LLM diets (diets 5.1 & 5.3). Similarly, serum AST values from birds fed LLM diets, and the mimosine supplemented diet (diet 5.8), were not significantly different from that in control birds. There was also no difference ($P>0.05$) between activity in birds fed the basal LLM diet and those fed the other diets.

3.3.6 Experiment 6.

A chick fed the control diet (diet 6.1) died during the 14d experiment and post mortem examination did not reveal the cause of death. Two chicks fed the LLM diet supplemented with the higher quantity of hemicellulase (diet 6.3), also died. One of these showed no signs as to the cause of death while the other was considered to have died because of chronic yolk sac infection. Other chicks remained healthy and active throughout the experiment. All chicks fed the treated LLM as the sole dietary component survived the experiment.

Table 3.3.20 Daily growth rate (GR) and food intake (FI) of chicks, efficiencies of food conversion (EFC) and nitrogen retention (ENR), and apparent metabolisable energy (AME) of diets in experiment 6.

	diet No.			sem
	6.1	6.2	6.3	(6 df)
GR (g chick^{-1} d^{-1})	19.98	10.50	10.29	0.8221
FI (g chick^{-1} d^{-1})	33.19	30.70	29.48	1.5270
EFC	0.602	0.342	0.349	0.01947
ENR	0.568	0.247	0.243	0.01983
AME(c) (MJ kg^{-1} DM)	15.34	8.76	9.13	0.2534
AME(n) (MJ kg^{-1} DM)	14.72	8.35	8.73	0.2231
AME(c)LLM (MJ kg^{-1} DM)	---	-1.19	-0.27	---
AME(n)LLM (MJ kg^{-1} DM)	---	-0.70	0.26	---

AME of the LLM was calculated from the AME of the diets as described in section 2.13.13.
GR, FI & EFC were determined from 7-21 days of age.
AME and ENR values were determined from 19-21days of age.

The AME(c) values for LLM obtained by feeding the LLM either solely (-1.49 MJ kg^{-1} DM) or as part of a diet (Table 3.3.20) were less than 0. This is reflected in the highly significant reduction in dietary AME value ($P<0.001$) of LLM diets (6.2 & 6.3) compared with that for the control diet (6.1). Inclusion of LLM caused a highly significant ($P<0.001$) depression in growth rate, EFC and ENR compared to the control diet (Table 3.3.20). Food intake however, was not significantly affected ($P>0.05$) by diet. The AME values calculated for LLM (Table 3.3.20) show a slight increase with the highest addition of hemicellulase (diet 6.3) but the difference is not significant ($P>0.05$) and the AME values are not significantly different from 0.

Daily mimosine and 3,4-DHP intakes for the LLM fed birds were 452 and 465 mg kg^{-1} liveweight for mimosine and 90.4 and 93.0mg kg^{-1} for 3,4-DHP intakes for chicks fed diets 6.2 and 6.3 respectively.

3.3.7 Experiment 7

Three chicks died during the experiment; one from each of the sorghum containing diets (diets 7.2, 7.3 & 7.4). All chicks were in good condition and post mortem did not reveal the cause of death. The other animals remained healthy and active throughout the 14d experimental period.

Consumption of the sorghum basal diet (7.2) did not significantly ($P>0.05$) affect the growth rate (Table 3.3.21) of chicks compared to those fed on the control diet (7.1). Addition of $Fe_2(SO_4)_3$ to the sorghum diet at 8g kg^{-1} (diet 7.3) significantly depressed growth rate ($P<0.05$) compared to control and sorghum basal diet fed birds. Further supplementation with Fe(III) depressed growth ($P<0.001$) further to about half that of the birds fed the control and basal sorghum diets. Addition of Fe(III) (diets 7.3 & 7.4) caused a severe depression in food intake ($P<0.001$) compared with control fed chicks. The highest inclusion rate of Fe(III) (diet 7.4) also caused a significant decrease in food intake compared with the chicks fed the sorghum diet (diet 7.2).

Inclusion of sorghum in the diet (diet 7.2) caused a slight ($P>0.05$) depression in food intake compared with control diet (7.1) fed chicks.

Birds fed the sorghum diet with the largest supplement of Fe(III) caused a highly significant ($P<0.001$) depression in EFC compared to birds fed the sorghum basal, and the control diets. A similar comparison for ENR shows a significantly higher ($P<0.05$) value for the diet with the largest amount of Fe(III) (7.4) .

Table 3.3.21 Efficiency of food conversion (EFC), efficiency of nitrogen retention (ENR), apparent metabolisable energy (AME) and daily growth rate (GI) and food intake (FI) of chicks in experiment 7.

	diet No. 7.1	7.2	7.3	7.4	sem (9 df)
GR (g chick^{-1} d^{-1})	25.24	25.40	20.00	12.20	1.5528
FI (g chick^{-1} d^{-1})	42.28	42.69	34.54	25.05	1.5804
EFC (weight gain/DM intake)	0.597	0.595	0.579	0.487	0.01567
ENR	0.655	0.635	0.657	0.713	0.01628
AME(c) (MJ kg^{-1} DM)	13.68	12.97	13.25	13.43	0.1625
AME(n) (MJ kg^{-1} DM)	12.91	12.23	12.49	12.62	0.1482

GR, FI and EFC were determined from 7-21 days of age. ENR and AME were determined from 18-21days of age.

Table 3.3.22 Efficiency of food conversion (EFC) and nitrogen retention (ENR), apparent metabolisable energy (AME) and daily growth rate (GR) and food intake (FI) of chicks fed jack bean diets.

	diet No. 9.1	9.2	9.3	9.4	9.5	sem (12df)
GR (g chick^{-1} d^{-1})	34.89	21.82	17.66	25.08	26.56	0.7550
FI (g chick^{-1} d^{-1})	45.85	32.04	27.72	36.35	37.83	0.9282
EFC (weight gain/DM intake)	0.761	0.681	0.637	0.690	0.702	0.01021
ENR	0.609	0.547	0.508	0.532	0.540	0.01711
AME(c) (MJ kg^{-1} DM)	14.15	14.08	14.14	14.13	13.97	0.07378
AME(n) (MJ kg^{-1} DM)	13.39	13.41	13.53	13.49	13.30	0.06121

GR, FI & EFC were determined from 7-19 days of age.
ENR & AME were determined from 16-19 days of age.

Dietary AME values (Table 3.3.21) were not significantly different (P>0.05).

3.3.8 Experiment 8.

All birds survived the experimental period and appeared active and healthy throughout the 7d duration of the experiment.

The mean daily DM intake for each chick, irrespective of diet, was 61.19g (SD=0.142) while the mimosine intake, for chicks fed the mimosine supplemented diet, was 235.7 (SD=5.91) mg kg^{-1} day^{-1}. The daily growth rate of chicks fed diets with or without added mimosine did not differ significantly (P>0.05) being 19.00 and 19.20 (sem [7 df] =1.0238) for birds fed the mimosine free and mimosine supplemented diets respectively. The EFC values for both diets were not affected (P>0.05) by the absence or presence of mimosine. Chicks fed the control diet and the diet containing mimosine had EFC values of 0.311 and 0.313 (sem [7 df] =0.01657) respectively. Values for ENR differed significantly (P<0.05), being lower (0.568) for birds fed the diet containing mimosine than for birds fed the control diet (0.620; sem [7 df] =0.01523). AME(c) for mimosine free and mimosine added diets were 14.22 and 14.32 MJ kg^{-1} DM (sem [7 df] = 0.07468) respectively; the differences not being significant (P>0.05). The absence or presence of mimosine had no effect (P>0.05) on the AME(n) values of the diets; 13.69 and 13.85 MJ kg^{-1} DM (sem [7 df] =0.2239) respectively.

3.3.9 Experiment 9.

There were no mortalities and all animals used in this experiment were active and healthy throughout the experimental period. The experiment was of 12 days duration because it was considered that there was insufficient food to last for 14 days.

Chicks fed autoclaved JB diets (9.2-9.5) had a markedly reduced growth rate compared with chicks fed the control diet (9.1; Table 3.3.22). Consumption of the JB basal diet (diet 9.2), caused a highly significant (P<0.001) depression in growth rate. Supplementation of the JB diet with lysine (diet 9.3) exacerbated the growth depression, which was significantly lower (P<0.01) than that of birds fed the JB basal diet (9.2). Addition of arginine and a mixture of arginine and lysine (diets 9.4 & 9.5 respectively) made a highly significant improvement (P<0.001) in growth rate of birds fed these diets compared with those fed the JB basal diet (9.2). Although growth rate improved considerably, there was still a large

Table 3.3.23 Serum canavanine, urea and ammonia concentrations (mg dm^{-3}), daily canavanine intake and the ratio of serum canavanine concentration to canavanine intake.

	Diet No.						
	9.1	9.2	9.3	9.4	9.5	sem	df
canavanine	0.00	14.19	10.25	12.02	11.10	1.2656	9
urea	10.58	20.90	27.73	45.78	42.53	4.1709	12
ammonia	9.08	12.53	11.55	11.23	12.83	1.1503	12
canavanine intake*	0.0	294.9	302.4	309.7	312.1	5.4103	9
serum/intake canavanine+	----	48.29	33.93	38.81	35.57	4.2945	9

* : mg kg^{-1} liveweight d^{-1}.
+ : [(ug dm^{-3})/(mg kg^{-1} liveweight d^{-1})
serum obtained from 19d old chicks.

discrepancy ($P<0.001$) between chicks on the control diet and those on the JB diets supplemented with arginine or arginine and lysine.

Food intake for all chicks fed the JB diets were significantly lower than those fed the control diet (diet 9.1). Addition of lysine (diet 9.3) depressed food intake significantly ($P<0.01$) compared to chicks fed the JB basal diet (diet 9.2). Addition of arginine (diet 9.4) on the other hand caused a significant increase in food intake compared with JB basal fed chicks. Food intake, compared with JB basal fed chicks, further increased $P<0.01$) when lysine and arginine was added to the JB diet (diet 9.5).

The JB basal diet caused a significantly lower ($P<0.001$) EFC than the control diet (diet 9.1). A larger reduction in EFC was associated with the JB diet containing supplemental lysine (diet 9.3); the EFC being significantly lower ($P<0.001$) than the control diet but not significantly less ($P>0.05$) than the basal JB diet. The arginine supplemented diet (9.4) caused a significantly reduced ($P<0.001$) EFC compared to the control diet as did the arginine/lysine supplemented diet ($P<0.001$). There was no significant difference ($P>0.05$) between chicks fed the JB diets, irrespective of supplementation.

The ENR values for chicks fed any of the JB diets were significantly lower ($P<0.05$) than that for the control diet but there was no difference ($P>0.05$) between values for any of the JB diets.

All the diets had AME (Table 3.3.22) values which did not differ significantly ($P>0.05$) from each other.

Supplementation of JB basal diet (diet 9.2) with lysine (diet 9.3) caused a small and non significant ($P>0.05$) reduction in the serum canavanine content compared to birds fed the JB basal diet (Table 3.3.23). Supplemental arginine and arginine plus lysine also caused a slight, but non significant ($P>0.05$), depression in serum canavanine levels compared to chicks fed the basal JB diet.

Serum urea concentrations are considerably influenced by the presence of JB in the diet (Table 3.3.23). The urea concentration in the serum of chicks fed the JB basal diet (diet 9.2) is almost twice that in the serum of chicks fed the control diet (diet 9.1) but the difference is not significant ($P>0.05$). Supplementary lysine caused an increase in serum urea above that found in birds fed the JB basal diet but the increased urea concentration was not significant ($P>0.05$). The higher urea concentration in birds fed the lysine supplemented diet (diet 9.3) was significantly higher ($P<0.05$) than that of control fed animals. Addition of

Table 3.3.24 Efficiency of food conversion (EFC) and nitrogen retention (ENR), apparent metabolisable energy (AME) and daily growth rate (GR), food intake (FI) and canavanine intakes, of chicks fed diets containing jack beans.

	diet No. 10.1	10.2	10.3	10.4	10.5	sem (12 df)
GR (g chick^{-1} d^{-1})	35.56	29.66	28.89	30.28	30.82	0.9438
FI (g chick^{-1} d^{-1})	45.94	40.52	39.74	41.59	42.45	1.0224
EFC	0.774	0.732	0.727	0.728	0.726	0.00696
ENR	0.568	0.566	0.523	0.522	0.514	0.01384
AME(c)(MJ kg^{-1} DM)	15.00	14.67	14.50	15.30	14.40	0.05715
AME(n)(MJ kg^{-1} DM)	14.22	13.88	13.78	14.59	13.71	0.05260
canavanine intake (mg kg^{-1} liveweight d^{-1})	0.0	289.2	289.6	293.8	294.7	1.9667*

* 9 df.

arginine and arginine and lysine to the JB diets (diets 9.4 & 9.5) caused a further substantial increase in serum urea concentrations. The concentrations of urea in the serum of birds fed these diets was much higher ($P<0.001$) than that of control fed birds. The urea concentrations in the serum of birds fed these diets (diets 9.4 & 9.5) were also significantly greater ($P<0.01$) than that of birds fed the basal JB diet.

Diets containing JB caused a slight increase in serum NH_3 concentrations compared to control fed birds. The NH_3 concentrations were significantly higher ($P<0.05$) for birds fed the JB basal diet and the JB diet supplemented with arginine and lysine (diet 9.5), than control fed birds. Birds fed diets containing JB did not differ significantly in their serum NH_3 levels.

There was a slight, but non significant increase ($P>0.05$) in canavanine intake, relative to liveweight, for chicks fed the JB diet supplemented with lysine (diet 9.3), compared with those fed the JB basal diet (Table 3.3.23). Although there was a small increase in canavanine intake between the chicks fed the JB diet supplemented with arginine (diet 9.4) and those fed the JB basal diet (diet 9.2), the increase was not significant. Consumption of the JB diet supplemented with lysine and arginine (diet 9.5) did produce a significant increase ($P<0.05$) in canavanine intake. When serum canavanine concentration is related to canavanine intake (Table 3.3.23), chicks fed the lysine supplemented JB diet (diet 9.3) and those fed the lysine and arginine supplemented diet (diet 9.5) had a significantly lower ($P<0.05$) serum to intake canavanine ratio than birds fed the JB basal diet.

3.3.10 Experiment 10.

One chick died during the experiment. The chick was in good bodily condition and death was attributed to broiler sudden death syndrome and was not associated with diet. Other animals appeared active and healthy throughout the 12 day experimental period.

Growth rate of chicks (Table 3.3.24) fed the JB basal diet (diet 10.2) is significantly lower ($P<0.001$) than that of birds fed the control diet (diet 10.1). Addition of ornithine to the JB diet (diet 10.3) caused a further slight depression of growth; this was not significant compared when with the growth rate of chicks fed the basal JB diet. Addition of arginine or lysine (diets 10.4 & 10.5) caused a slight, but not significant ($P>0.05$), improvement in growth rate however growth rate for chicks fed these diets remained significantly lower ($P<0.01$) than chicks fed the control diet.

Food intake for chicks fed the JB diets was significantly lower ($P<0.05$) than chicks fed the control diet (diet 10.1). Supplementary amino acids to the JB diets did not significantly increase ($P>0.05$) food intake compared to chicks fed the JB basal diet (diet 10.2).

The EFC value (Table 3.3.24) for the JB basal diet is significantly lower ($P<0.01$) than that for the control diet. The amino acid supplemented diets also had lower EFC values ($P<0.001$) than the control diet but there were no significant ($P>0.05$) differences in the EFC values of the JB diets.

The JB basal diet did not have a significantly different ENR ($P>0.05$) than the control diet. The ornithine supplemented diet (diet 10.3) had an appreciably lower ($P<0.05$) ENR than control fed birds but there was no difference between this diet (diet 10.3) and the JB basal diet (diet 10.2). The arginine supplemented diet (diet 10.4) had a considerably lower ($P<0.05$) ENR than both the control and JB basal diets while lysine supplementation caused a further depression in ENR, being significantly lower than the control ($P<0.01$) and the JB basal diet ($P<0.05$).

There is considerable variation in AME values of the diets (Table 3.3.24). The AME(c) value of the JB basal diet is substantially lower ($P<0.01$) than that of the control diet. The AME(c) of the arginine supplemented diet (10.4), on the other hand, is significantly higher ($P<0.01$) than that of the control diet. The other two JB diets (10.3 & 10.5) had much lower ($P<0.001$) AME(c) values than the control diet. The arginine and lysine supplemented diets had significantly higher and lower ($P<0.01$) AME(c) values respectively, than the JB basal diet (10.2).

Dietary AME(N) values follow the same trend as AME(c) values. All the diets containing JB have values which are significantly different ($P<0.001$) from the control diet. The diet supplemented with arginine (diet 10.4) had a significantly larger ($P<0.001$) AME than the JB basal diet while that with additional lysine was significantly lower ($P<0.05$).

Daily canavanine intake (Table 3.3.24) increased slightly with the addition of ornithine, arginine and lysine to the diets. The diet with additional ornithine, arginine and lysine (diet 10.5) caused a significant ($P<0.05$) increase in canavanine intake when compared with the JB basal diet.

Table 3.3.25 Efficiency of food conversion (EFC) and nitrogen retention (ENR), apparent metabolisable energy (AME), daily growth rate (GR) and food intake (FI) of chicks in experiment 11.

diet No.	GR (g chick^{-1} d^{-1})	FI	EFC	ENR	AME(c) (MJ kg^{-1} DM)	AME(N)
11.1	32.87	45.34	0.725	0.730	15.10	14.15
11.2	24.11	33.63	0.717	0.684	14.72	13.82
11.3	6.85	15.36	0.446	0.630	14.14	13.37
11.4	5.16	13.54	0.381	0.654	14.37	13.51
11.5	28.44	43.22	0.658	0.647	14.14	13.34
11.6	29.64	42.16	0.703	0.711	14.82	13.82
11.7	23.23	35.04	0.663	0.672	14.32	13.43
11.8	6.14	14.21	0.432	0.588	14.17	13.41
11.9	8.86	18.89	0.469	0.667	14.64	13.80
11.10	8.81	18.05	0.488	0.582	13.86	13.11
sem (27 df)	1.3855	1.3874	0.03310	0.02377	0.2047	0.1847

GR, FI & EFC were determined from 7-19 days of age.
ENR and AME were determined from 14-19 days of age.

3.3.11 Experiment 11.

Three birds in this experiment died during the 12 day experimental period. The birds which died were from different diets and in good bodily condition; the cause of death could not be determined and could not be attributed to diet. Salmonella infection was identified in these chicks.

As in previous experiments reported here the JB basal diet (diet 11.2) caused a substantial growth depression ($P<0.001$) compared with the growth rate of control (diet 11.1) fed birds (Table 3.3.25). Growth depression was most acute ($P<0.001$) for birds fed diets (diets 11.3, 11.4, 11.8, 11.9 & 11.10; Table 3.3.25) supplemented with JB at a level of 280g kg^{-1} compared with chicks fed either the control (diet 11.1) or the JB basal diet (diet 11.2). Growth depression of birds fed the diet (diet 11.5) containing the JB residue, after extraction of canavanine, at 280g kg^{-1} was significantly lower ($P<0.05$) than control fed birds but much less severe than the birds fed the other diets containing JB at 280g kg^{-1}. Birds fed the diet containing the residual material grew faster ($P<0.05$) than birds fed the JB basal diet. Chicks fed the canavanine supplemented diet (diet 11.6) grew slightly more slowly than birds fed the control diet although the difference was not significant ($P>0.05$). Animals fed the canavanine diet (diet 11.6) did, however, grow faster ($P<0.01$) than those fed the basal JB diet (diet 11.2).

Germination of beans (diets 11.3 & 11.4) had no beneficial effect ($P>0.05$) on growth rate compared with chicks fed beans treated in other ways and included at 280g kg^{-1}. Drying JB at 100 C, prior to dietary inclusion at 140g kg^{-1} (diet 11.7), did not beneficially affect growth ($P>0.05$) of birds compared to those fed the basal JB diet. There were no significant differences ($P>0.05$) in the growth of chicks fed diets containing JB which had been subjected to the various heat treatments (Table 2.13.19).

Only chicks fed the diets containing the extracted residue (diet 11.5) and added canavanine (diet 11.6) had food intakes which were essentially the same ($P>0.05$) as control fed birds (diet 11.1). Chicks fed the other JB diets had food intakes which were significantly lower ($P<0.001$) than control fed chicks. The diets containing the extracted JB and added canavanine (diets 11.5, 11.6) were consumed at a significantly higher rate ($P<0.001$) than the diets containing JB treated in other ways.

The EFC values obtained, varied widely between diets (Table 3.3.25). The highest EFC obtained was that of the control diet (diet 11.1). The basal JB diet had a slightly lower EFC than that of the control diet but the difference was not

Table 3.3.26 Serum canavanine, ammonia and urea concentrations (mg dm^{-3}), and serum activity of aspartate amino transferase (AST) and alanine amino transferase (ALT).

Diet No.	canavanine	NH_3	urea	AST	ALT
	(--------mg dm^{-3}----------)			*(units dm^{-3} at 37°C)	
11.1	0.00	11.75	14.02	167.6	9.84
11.2	5.75	13.60	18.55	164.5	15.76
11.3	6.00	11.52	29.65	147.5	13.33
11.4	7.16	13.27	18.32	136.6	10.76
11.5	2.33	10.77	10.02	141.2	7.99
11.6	74.08	9.20	17.70	157.2	7.30
11.7	4.53	12.57	29.45	134.23	9.69
11.8	3.08	10.85	17.82	148.8	6.85
11.9	18.38	9.72	19.87	126.1	12.09
11.10	10.93	11.77	23.00	164.8	7.95
sem	2.5722	1.3875	4.1278	18.2759	2.3328
(df)	(23)	(27)	(27)	(25)	(23)

* An activity of 1 unit dm^{-3} is defined as a change in absorbance of 0.001 absorbance units min^{-1} at 340nm using the procedure described (sect. 2.11) serum obtained from 19 day old chicks.

Table 3.3.27 Daily canavanine intake and the ratio of serum canavanine concentration to canavanine intake.

Diet No.	canavanine intake+	serum/intake canavanine*
11.1	0.0	-----
11.2	220.2	26.51
11.3	650.7	9.21
11.4	589.5	10.71
11.5	28.6	83.96
11.6	321.9	229.22
11.7	208.9	21.58
11.8	404.4	7.45
11.9	431.3	42.65
11.10	208.7	53.18
sem(df)	14.3292 (24)	13.4777 (23)

+ expressed as mg kg^{-1} liveweight d^{-1}.
* expressed as ug dm^{-3}/ mg kg^{-1} d^{-1}.

significant (P>0.05). Birds fed any of the diets containing JB at a level of 280g kg^{-1} had a highly significant reduction (P<0.001) in EFC compared to control or JB basal diet fed birds. Inclusion of the JB residue (diet 11.5) after extraction of canavanine, cuased a small but insignificant (P>0.05) depression in EFC compared to the control or JB basal diets. Supplemental canavanine in the JB free diet (diet 11.6) also caused a minor depression (P>0.05) in EFC in comparison to control and basal JB diets. Heating JB at 100°C prior to dietary inclusion (diet 11.7) caused a depression in EFC, which was not significant (P>0.05), compared with those for the control and JB basal diets.

The chicks fed the control diet had the highest ENR values (Table 3.3.25). The ENR value for chicks fed the JB basal diet was lower than chicks fed the control diet although the difference was not significant (P>0.05). Inclusion of 3 day germinated beans (diet 11.3) reduced ENR compared to the control diet (P<0.01) as did (P<0.05) the inclusion of the 5 day germinated beans (diet 11.4) and the JB residue (diet 11.5) in the diets. The JB heated to 100°C and included in the diets (diets 11.8 & 11.10) at 280g kg^{-1} caused a highly significant depression (P<0.001) in ENR compared to the control diet. These two diets (diets 11.8 & 11.10) were the only two which had significantly different (P<0.01) ENR values from the JB basal diet.

The AME(c) values (Table 3.3.25) of the diets which included germinated JB (diets 11.3 & 11.4) and the extracted residue (diet 11.5) at 280g kg^{-1}, were significantly lower (P<0.01) than the control diet. The three diets (11.7, 11.8 & 11.10) that incorporated JB which had been heated to 100°C were also significantly lower (P<0.01) than the control; diet 11.10 being the lowest (P<0.001). Only the diet (diet 11.10) containing minced JB and heated to 100°C showed a significantly reduced (P<0.01) AME(c) compared to the JB basal diet (diet 11.2). The AME(n) values of the diets showed an identical trend to those for AME(c).

There is considerable variation in the serum canavanine contents of chicks fed the various JB diets (Table 3.3.26). The most obvious difference in the serum canavanine content is that for chicks fed the diet with added canavanine (diet 11.6), which is about 12 fold higher than that for chicks fed the basal JB diet (diet 11.2) and thus is highly significant (P<0.001). Of the other diets fed, only the diet (diet 11.9) containing minced beans, dried at 60°C and included at 280g kg^{-1}, produced serum canavanine levels which were significantly higher (P<0.01) than those of the JB basal fed birds. Chicks fed the diet (diet 11.5) containing the extracted JB residue had the lowest serum canavanine content although it was not significantly lower (P>0.05) than the JB basal fed birds. Feeding beans which

were dried at 100°C appears to have had a small effect in reducing the canavanine concentration (diets 11.7 & 11.8) although drying minced beans at 100°C, did not decrease, but caused a slight increase in the serum canavanine level (diet 11.10).

Serum NH_3 levels (Table 3.3.26) approximate to 10mg dm^{-3}, there being no difference ($P>0.05$) between the level in serum from control fed birds and JB fed birds. Chicks fed the diet containing added canavanine (diet 11.6), had significantly lower serum NH3 levels than birds fed the JB basal diet. Chicks fed the diet containing the extracted JB residue (diet 11.5) had a slightly lower ($P>0.05$) serum urea concentration (Table 3.3.26) than birds fed the control diet. Chicks fed diets containing JB and canavanine all had higher serum urea levels than control fed birds however only chicks fed the diet (diet 11.3) containing the 3 day germinated beans and that containing ground beans dried at 100°C (diet 11.7), had significantly higher ($P<0.05$) serum urea levels than control fed birds. There was no significant differences between the serum urea levels of chicks fed the JB basal diet and those fed the other JB or canavanine supplemented diets.

Although there was some variation in serum AST activity (Table 3.3.26) there were no significant differences ($P>0.05$) between the activity in the serum of any of the chicks fed any of the diets. There was no significant difference ($P>0.05$) in serum ALT activity between chicks fed the control diet and those fed the JB and canavanine supplemented diets. There are, however, significantly lower ($P<0.05$) activities for chicks fed the diets containing JB residue (diet 11.5), additional canavanine (diet 11.6) and JB dried at 100°C and included at 280g kg^{-1} (diets 11.8 & 11.10), compared to those fed the basal JB diet (diet 11.2).

Mean daily canavanine intake, relative to liveweight, (Table 3.3.27) varied widely depending on diet fed. Chicks which consumed diets containing JB at a level of 280g kg^{-1} (diets 11.3, 11.4, 11.8 & 11.9), with the exception of the diet (diet 11.10) containing minced JB dried at 100°C, had very high canavanine consumptions which were significantly greater than the JB basal fed birds. The chicks fed the canavanine supplemented diet (diet 11.6) also had a significantly increased consumption ($P<0.01$) of canavanine relative to the basal JB fed birds. Incorporation of the residual JB into a diet (diet 11.5) caused an appreciably lower ($P<0.001$) intake of canavanine with respect to the JB basal diet.

When serum canavanine is related to canavanine intake (Table 3.3.27) results vary widely. Chicks fed the canavanine supplemented diet (diet 11.6) had the highest amount of canavanine in the serum relative to the canavanine intake ($P<0.001$), when compared with birds fed the JB basal diet. Ingestion of the diet

Table 3.3.28 Efficiency of food conversion (EFC) and nitrogen retention (ENR), apparent metabolisable energy (AME), daily growth rate (GR) and food intake (FI) of chicks in experiment 12.

diet No.	GR (g chick^{-1} d^{-1})	FI	EFC	ENR	AME(c) (MJ kg^{-1} DM)	AME(N)
12.1	31.72	43.69	0.726	0.748	15.01	13.99
12.2	19.78	31.3	0.632	0.644	13.89	13.02
12.3	26.27	39.03	0.673	0.598	13.49	12.69
12.4	26.59	38.65	0.688	0.696	14.44	13.54
12.5	25.37	37.14	0.683	0.661	13.98	13.11
sem (12 df)	0.9210	1.0203	0.02024	0.02557	0.2558	0.2248

Table 3.3.29 Serum canavanine, ammonia and urea concentrations (mg dm^{-3}), and serum activity of aspartate amino transferase (AST) and alanine amino transferase (ALT).

Diet No.	canavanine (mg dm^{-3})	NH3	urea	AST *(units dm^{-3}	ALT at 37°C)
12.1	0.00	11.43	8.92	191.0	9.84
12.2	6.98	12.45	12.57	163.7	8.21
12.3	2.13	14.65	34.05	142.2	7.32
12.4	4.28	10.20	28.62	173.5	8.40
12.5	6.78	10.20	37.07	139.3	9.36
sem (12 df)	1.2947(9 df)	1.5870	3.4455	13.2440	1.1987

* An activity of 1 unit dm^{-3} is defined as a change in absorbance of 0.001 absorbance units min^{-1} at 340 nm using the procedure described previously (sect 2.11).
serum samples obtained from 19d old chicks.

(diet 11.5) with the extracted JB caused a higher serum canavanine level, in relation to canavanine intake ($P<0.01$), than the JB basal diet. The ratios for serum canavanine and canavanine intake for chicks fed the other diets did not vary significantly ($P>0.05$) from the value obtained for the JB basal diet. Chicks fed the germinated beans had a lower serum canavanine concentration relative to intake, than birds fed the JB basal diet but the difference was not sufficiently large as to be significant ($P>0.05$).

3.3.12 Experiment 12.

All birds appeared healthy and active and survived the 12 day experimental period.

A severe ($P<0.001$) growth depression was observed when chicks were fed the JB basal diet (diet 12.2) rather than the control diet (Table 3.3.28). The lowest level of supplementation of arginine and lysine (diet 12.3) was as effective as the higher levels of supplementation (diets 12.4 & 12.5; Table 2.13.22) of arginine and lysine at increasing growth rate ($P>0.05$). Growth rates of chicks fed the diets supplemented with the two lower levels of arginine and lysine (diets 12.3 & 12.4) were still considerably lower ($P<0.01$) than the control fed birds. The diet containing the largest supplement of arginine and lysine (diet 12.5) responded least well; growth rates remaining well below ($P<0.001$) the control fed birds. Conversely, the most significant improvement ($P<0.001$) in growth rate, compared to the chicks fed the JB basal diet, was observed with birds fed the diets containing the lower levels of arginine and lysine (diets 12.3 & 12.4).

Food intake for chicks fed the JB basal diet (diet 12.2) and the diet supplemented with the highest amount of arginine and lysine (diet 12.5), was much lower ($P<0.001$) than chicks fed the control diet (diet 12.1). The intake of diets supplemented with lower amounts of arginine and lysine (diets 12.3, 12.4) were also lower ($P<0.01$) than the control diet. The diets supplemented with lower levels of arginine and lysine (diets 12.3, 12.4) caused higher intakes ($P<0.001$) than the JB basal diet. Consumption of the diet supplemented with the greatest amount of arginine and lysine was also significantly higher ($P<0.01$) than the JB basal diet.

The EFC value of the basal diet (diet 12.2; Table 3.3.28) was significantly lower ($P<0.01$) than the control diet, however the other diets were only slightly lower ($P>0.05$) than the control. The arginine and lysine supplemented diets had higher EFC values than the JB basal diet but the difference was not significant ($P>0.05$).

Table 3.3.30 Daily canavanine intake and the ratio of serum canavanine concentration to canavanine intake.

diet No.	canavanine intake+	serum/ intake canavanine*
12.1	0.0	----
12.2	225.2	31.27
12.3	232.1	9.16
12.4	227.9	18.93
12.5	227.0	29.86
sem (9 df)	3.0759	5.8469

+ : mg kg^{-1} liveweight d^{-1}
* : ug dm^{-3}/ mg kg^{-1} liveweight d^{-1}.

Chicks fed the JB basal diet and the diet with the largest supplement of arginine and lysine (diet 12.5), utilised dietary nitrogen significantly less efficiently ($P<0.05$) than birds fed the control diet (Table 3.3.28). The lowest ENR, which was significantly lower ($P<0.01$) than the control, was obtained for the diet containing the lowest quantity of added arginine and lysine (diet 12.3). The ENR of the supplemented JB diets were not significantly different ($P>0.05$) from that obtained for the JB basal diet.

The AME(c) and AME(N) values of the control diet were significantly higher ($P<0.05$) than all of the JB diets except for that with intermediate supplementation (diet 12.4) of arginine and lysine ($P>0.05$). There was no difference ($P>0.05$) between the AME values for the JB diets.

Inclusion of arginine and lysine into JB diets had little effect on the serum canavanine content (Table 3.3.29) of chicks fed these diets; only the diet containing the lowest supplemental arginine and lysine (diet 12.3) produced significantly lower ($P<0.05$) serum canavanine levels than in the birds fed the basal JB diet. It is worth noting that the concentrations of canavanine in serum in this experiment are about half of those in experiment 9 (Table 3.3.23).

Diet had no effect ($P>0.05$) on the serum NH_3 concentrations of chicks in this experiment however there was considerable variation in urea concentrations in serum (Table 3.3.29). The serum urea concentration of the chicks fed the JB basal diet (diet 12.2), although somewhat higher, is not significantly different ($P>0.05$) from that of control fed birds. Diets supplemented with lowest (diet 12.3) and highest (diet 12.5) amounts of arginine and lysine show significant increases ($P<0.001$) in serum urea compared to control fed birds. The intermediate level of supplementation with arginine and lysine (diet 12.4) did not produce such a large increase in serum urea compared with the control diet although the increase was significant ($P<0.01$). Comparison of urea levels of chicks fed the JB basal diet with those of chicks fed the arginine and lysine supplemented diets, shows a highly significant increase ($P<0.001$) for the low (diet 12.3) and high (diet 12.5) levels of supplementation. Intermediate level of supplementation with arginine and lysine (diet 12.4) also gave a significant ($P<0.01$) increase in serum urea compared to basal fed birds. The concentration was lower, however, than birds fed the diets with low and high supplemental arginine and lysine.

There was no difference ($P>0.05$) between serum ALT activity (Table 3.3.29) of birds fed the diets containing JB and those fed the control diet although the activity in the control serum was slightly higher than for the JB fed birds. Chicks

Table 3.3.31 Efficiency of food conversion (EFC), and nitrogen retention (ENR), daily growth rate (GR) and food intake (FI) of chicks in experiment 13.

diet No.	GR	FI	EFC	ENR
	(g chick^{-1} d^{-1})			
13.1	34.77	46.42	0.749	0.572
13.2	31.86	45.45	0.701	0.423
13.3	26.63	42.49	0.622	0.381
13.4	35.09	49.56	0.708	0.362
13.5	25.90	44.05	0.588	0.256
sem (12 df)	1.0397	1.2223	0.01085	0.02431

GR, FI & EFC were determined from 7-19 days of age.
ENR was determined from 16-19 days of age.

Table 3.3.32 Apparent metabolisable energy (AME) values for lupin seed and diets.

diet No.	AME(c)	AME(n)	AME(c)	AME(n)
	diets		seeds	
	(MJ kg^{-1} DM)			
13.1	14.35	13.63	-----	-----
13.2	13.45	12.75	10.69	10.80
13.3	12.96	12.16	11.53	11.54
13.4	13.30	12.55	9.97	9.83
13.5	12.36	11.84	10.25	10.75
sem	0.09508	0.10151	0.41397	0.36188
(df)	(12)	(12)	(9)	(9)

period of collection: 16-19 days of age.
AME of the seeds calculated from the AME of the diets.

fed the JB basal diet had similar (P>0.05) serum ALT activities to chicks fed the arginine and lysine supplemented diets. Chicks fed diets with lowest (diet 12.3) and highest (diet 12.5) supplemental arginine and lysine had significantly lower (P<0.05) serum AST activity than control fed birds. Similarly to ALT activity, serum from control fed birds had the highest AST activity. There was no significant difference (P>0.05) in the AST activity in the serum of birds fed diets containing JB although birds fed diets with lowest and highest supplements of arginine and lysine, had lowest activities.

In this experiment diet did not affect canavanine intake when normalised to equal body weights (Table 3.3.30). The serum canavanine, related to normalised intake (Table 3.3.30), was highest for the JB basal fed chicks (diet 12.2) Although there was considerable variation in the values obtained for chicks fed the different arginine and lysine supplemented diets, the diet supplemented with the lowest amount of these amino acids (diet 12.3) had a significantly lower (P<0.01) ratio of serum canavanine relative to intake, than the birds fed the JB basal diet. The values of serum to intake canavanine increased, with increasing supplementation of arginine and lysine, to essentially the same as that of the JB basal fed chicks.

3.3.13 Experiment 13.

There were no mortalities throughout the 12 day experimental period during which all the animals appeared active and healthy.

Mean daily growth rate of chicks (Table 3.3.31) fed the reference diet was significantly higher (P<0.05) than those fed the diet containing uncooked lupin seed (diet 13.2) at a level of 200g kg^{-1} (Table 2.13.24). The equivalent diet (diet 13.4) with cooked lupin seed, produced a growth rate which was slightly higher than birds fed the reference diet but the increase was not significant (P>0.05). The difference in growth rate between the chicks fed the diets containing uncooked (diet 13.2) or cooked (diet 13.4) lupin seed was significant (P<0.05). Chicks fed the two lupin diets with 400g kg^{-1} of uncooked (diet 13.3) and cooked (diet 13.5) lupin seed grew at a much slower rate (P<0.001) than those fed the reference diet (diet 13.1). There was no significant difference (P>0.05) in rate of growth between chicks fed the diets containing 400g kg^{-1} lupin seed although chicks fed the cooked lupin diet (diet 13.5) grew slightly less rapidly than those fed the equivalent diet with uncooked lupin seed (diet 13.3). Chicks fed both diets containing lupin seed at 400g kg^{-1} grew less rapidly (P<0.01) than those fed the diet containing the unheated lupin seed (diet 13.2).

Although food intake varied slightly between diets the, intakes for lupin diets were not significantly different from the control diet (diet 13.1). The food intake for the diet containing cooked lupin at 200g kg^{-1} (diet 13.4) was higher than those containing the cooked or untreated lupin at 400g kg^{-1} (diets 13.3, 13.5).

The EFC of the reference diet was higher than those diets with lupin seed (Table 3.3.31). Inclusion of uncooked lupin seed at 200g kg^{-1} significantly ($P<0.01$) depressed EFC while the cooked lupin seed included at this level slightly, but not significantly ($P>0.05$), depressed EFC compared to birds fed the reference diet. The depression in EFC was highly significant ($P<0.001$) when lupin seed, which was cooked or uncooked, was included at a level of 400g kg^{-1} (diets 13.3 & 13.5), compared to both the reference diet and the diet (13.2) containing the uncooked lupin seed at 200g kg^{-1}.

Inclusion of lupin seed into the diets reduced the ENR values of the diets considerably compared to the reference diet (Table 3.3.31). Both diets (diets 13.2 & 13.3) containing the uncooked lupin seed as well as that containing cooked lupin at 400g kg^{-1} (diet 13.5), severely depressed ENR values ($P<0.001$) compared to the reference diet. The diet (diet 13.4) containing the lower level of cooked lupin (diet 13.4) also had a reduced ENR compared to the reference diet but the difference was less significant ($P<0.01$). Although the diet with the highest supplement of uncooked lupin seed (diet 13.3) had a lower ENR than the diet with 200g kg^{-1} of untreated lupin seed (diet 13.2), only the diet supplemented with 400 g of cooked lupin seed (diet 13.5) reduced ENR significantly ($P<0.001$) relative to the diet with 200g untreated lupin seed (diet 13.2).

Dietary AME(c) and AME(n) values are shown in table 3.3.32 and as expected inclusion of lupin seed (Table 2.13.24) reduced AME with respect to the reference diet; the depression being highly significant ($P<0.001$). The reduction in AME was directly related to inclusion level of lupin.

Inclusion of cooked lupin at a level of 400g kg^{-1} (diet 13.5) significantly reduced dietary AME, when compared with the diet (13.3) containing the same quantity of uncooked lupin. There was no significant ($P>0.05$) difference between the AME values of diets containing 200g kg^{-1} of cooked or uncooked lupin although that containing the cooked lupin was slightly lower.

There were no significant differences ($P>0.05$) between the calculated AME(c) values obtained for different inclusion levels of cooked or uncooked lupin seed although in both cases supplementation at the higher level produced slightly

Fig. 3.3.1 Typical HPLC chromatograms of lupin carbohydrates.
a & c: untreated lupin chromatographed on HP-87P and Nucleosil -5-NH_2 columns respectively.
b & d: enzyme treated lupin chromatographed on HP-87P and Nucleosil -5-NH_2 columns respectively.
Conditions as reported by Sutherland & Ford 1986.

I mannose
II galactose
III oligosaccharides

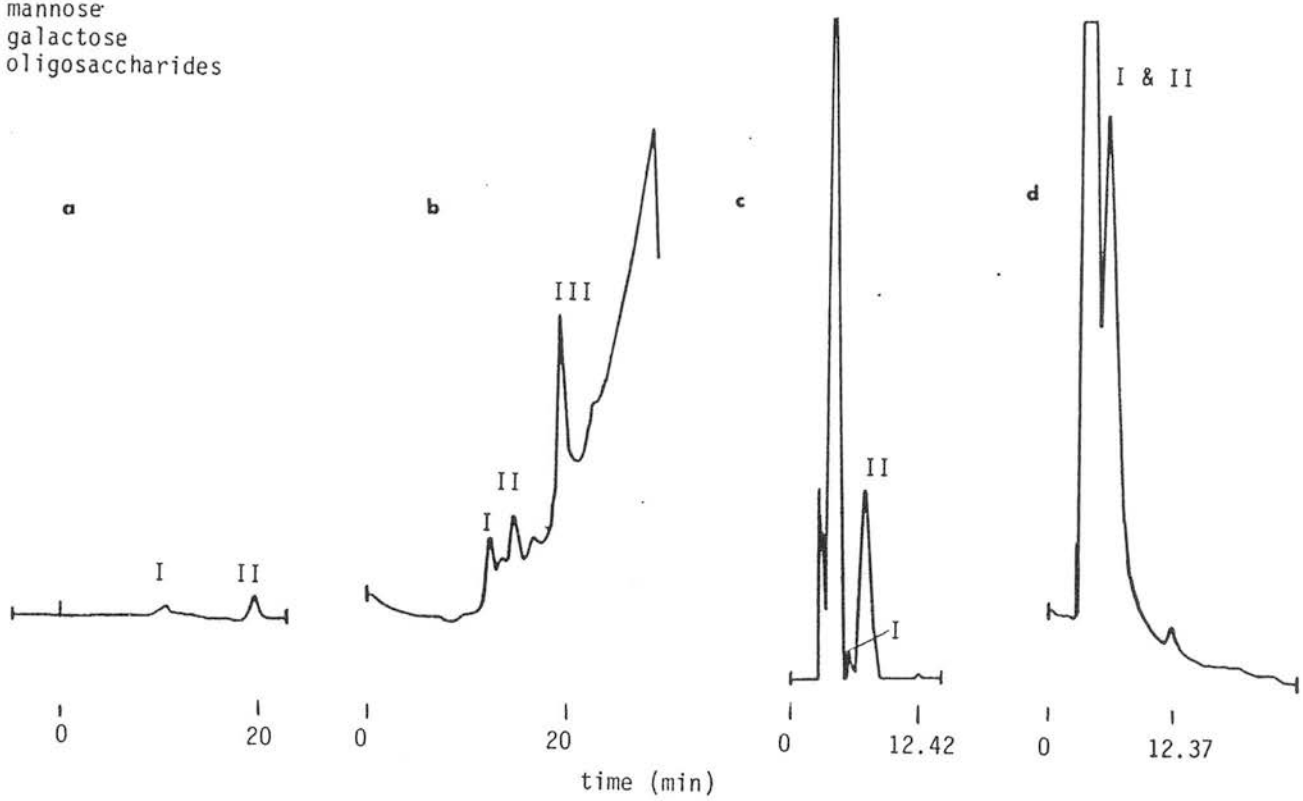


Table 3.3.33 Efficiency of food conversion (EFC) and nitrogen retention (ENR), apparent metabolisable energy (AME), daily growth rate (GR) and food intake (FI) of chicks in experiment 14.

diet No.	GR	FI	EFC	ENR	AME(c)	AME(N)
	(g chick^{-1} d^{-1})				(MJ kg^{-1} DM)	
14.1	25.26	41.01	0.616	0.655	13.68	12.91
14.2	22.80	38.97	0.585	0.603	13.10	12.26
14.3	22.66	46.06	0.492	0.582	13.39	12.56
14.4	17.80	35.67	0.499	0.514	13.83	13.19
14.5	18.32	37.70	0.486	0.443	13.11	12.54
sem (12 df)	1.1657	1.0976	0.04512	0.03920	0.20031	0.17662

higher values. The means of the AME(c) values for cooked and uncooked showed that the cooked lupin seeds had a significantly lower ($P<0.05$; unpaired t-test) AME(c) than uncooked seeds. Correction of AME for nitrogen retention showed (Table 3.3.32) that the value obtained for inclusion of cooked lupins at the higher level (diet 13.5) was significantly higher ($P<0.05$) than for inclusion at the low level (diet 13.4).

3.3.14 Experiment 14.

Two chicks fed different diets died during the 14 day experimental period. Both birds were in good bodily condition and cause of death could not be ascertained nor attributed to diet. Other chicks appeared active and healthy throughout the experimental period.

Growth rate of chicks fed the lupin basal diet (diet 14.2) and the lupin diet (diet 14.3) with supplemental dry enzyme mixture (Table 3.3.33), were depressed compared to control fed birds but the depression was not significant ($P>0.05$). Inclusion of enzyme treated lupin (3.8g enzyme kg^{-1} lupin), after drying (diet 14.4), caused a severe growth depression ($P<0.001$) compared with control fed birds. Lupin pretreated with a larger quantity of enzyme (7.6 g enzyme kg^{-1} lupin) and dried prior to incorporation into the diet (diet 14.5) also depressed growth rate ($P<0.01$) compared with the control diet. Growth of birds fed the enzyme treated lupin diets was significantly lower ($P<0.05$) than birds fed the lupin basal diet (diet 14.2).

Although all the lupin diets depressed food intake, only those (diets 14.4, 14.5) containing preatreated lupin, caused a significant depression ($P<0.001$ & 0.01 respectively). The diet containing the lupin pretreated with the lower amount of enzymes (diet 14.4) caused a significant ($P<0.01$) depression in intake compared to the basal lupin diet (diet 14.1).

The EFC values (Table 3.3.33) of diets supplemented with dry enzyme (14.3) and those with lupins, pretreated with enzyme (14.4 & 14.5), were lower than the control diet, however the decreased EFC values were not significantly different ($P>0.05$) from that of the control diet (14.1) or the basal diet (14.2).

Low level enzyme pretreatment and drying of lupins caused a significantly lower ($P<0.05$) ENR than the control diet (Table 3.3.33). The ENR was decreased further ($P<0.001$) with treatment with a larger quantity of enzyme (diet 14.5); the ENR of this diet was also significantly lower ($P<0.01$) than the basal lupin diet (14.2).

Dietary AME(c) values (Table 3.3.33) varied slightly, but not significantly ($P>0.05$). It is worth noting, however, that low level enzyme treatment of lupins (diets 14.3 & 14.4) caused a slight improvement in AME compared with the basal lupin diet. The nitrogen corrected AME value of the basal diet was significantly lower ($P<0.05$) than that of the control diet. Inclusion of lupin, treated with the low level of enzyme pretreatment, enhanced the dietary AME(n) value such that it was significantly larger ($P<0.01$) than the basal lupin diet.

CHAPTER 4

DISCUSSION

4.1 Analysis of mimosine and 3-hydroxy-4(1H)-pyridone.

The use of HPLC has decisively improved the qualitative and quantitative analysis of mimosine and 3,4-DHP as well as yielding a technique with potential for the study of metabolites of these.

The HPLC methods developed during the course of the work and presented here, show that mimosine and 3,4-DHP can be analysed accurately, rapidly with high specificity and sensitivity using UV or EC detection.

4.1.1 Analysis using HPLC and UV detection.

The use of reversed-phase ion-pair HPLC allows the simultaneous analysis of 3,4-DHP and mimosine with good resolution (Fig. 3.1.2). Strict control of pH of the eluent is necessary, while the use of octyl sodium sulphate and sodium nitrate produce a very versatile system which coped well with a variety of samples prepared by differing techniques. Increase in pH caused retention time to increase and bad peak shape to occur while variation in either octyl sulphate or sodium nitrate concentration allowed retention times to be varied readily. It is worth mentioning that the use of sodium dodecyl sulphate, as the ion pairing agent, caused extremely long retention times to the extent that frequently peaks never eluted from the column. Manufacturers of HPLC equipment and others (Rabel, 1980) have reported that Cl^- anions have an adverse and degradative effect on HPLC systems while HNO_3 has a passifying effect (Rabel, 1980). The inclusion of NO_3^- ions in the eluent, therefore, has the double benefit of acting as an ionic strength modifier and a passifier of the stainless steel within the HPLC system.

As has been stated earlier, the detector response to both mimosine and 3,4-DHP shows very good linearity and close correlation over the concentration ranges used. The linear working detection range for both mimosine and 3,4-DHP correspond well with that found recently by other workers (Lyon, 1985; Lowry, et.al. 1985) and is at least 10 fold more sensitive than the automated colorimetric procedure (Megarrity, 1978) which estimates mimosine and 3,4-DHP together as 'mimosine'. The lengthy and elaborate paper chromatographic method which was developed for the simultaneous estimation of mimosine and 3,4-DHP (Hegarty, Court and Thorne, 1964) is 100 fold less sensitive than the HPLC method

developed here while the cellulose electrophoretic method (Reis, Tunks and Hegarty, 1975) is about three orders of magnitude less sensitive. The GC method which has been described (Mee and Brooks, 1971) is also considerably less sensitive than the HPLC method described here. There is no indication regarding response to mimosine or to recovery using the GC technique, although recoveries may be similarly quantitative to those of other amino acids analysed using this technique (Roach and Gehrke, 1969). It was suggested that an internal standard be incorporated in the sample to account for losses and to quantify mimosine. This is not always recommended however because of the physical differences between the standard and the analytes, and the difference in the characteristics of the various analytes (De Leenheer and Nelis, 1981; Hart and White, 1986). The IEC methods used for the estimation of mimosine (Reis, Tunks and Chapman, 1975; Mzik, 1977, Acamovic and D'Mello, 1981) are also less sensitive by about three orders of magnitude, than the HPLC technique described here and in the literature (Acamovic, D'Mello and Fraser, 1982). The HPLC methods of other workers also show a similar increase in sensitivity (Tangendjaja and Wills, 1980; 1983; Lowry, Tangendjaja and Cook, 1985).

Detection limits, using HPLC and UV detection, are much lower than the concentration ranges employed here but baseline noise and variation make reproducibility a problem and therefore it is impracticable to work quantitatively, at levels much below the concentration ranges used here. Within these ranges mimosine and 3,4-DHP in *leucaena* samples can be readily and quantitatively determined.

Linearity of response, for the method used here, was equally good at each wavelength. The wavelength used for detection of both mimosine and 3,4-DHP however causes reduced sensitivity for one or other or, perhaps both, depending on which compromise is chosen. In samples where these compounds exist in small quantities close to the detection limits, the use of photodiode array, mutiwavelength or switchable wavelength detectors would overcome this disadvantage. If there are constraints on quantity of sample or 3,4-DHP, detection at 269nm is preferable since mimosine is more sensitively detected than 3,4-DHP simply because it has a smaller k' value than 3,4-DHP.

The large increase in the size of the early eluting peaks of serum from birds fed LLM (Fig. 3.1.2c) compared to that from birds fed an LLM free diet, may be due to flavonol glycosides from the LLM (Lowry, Cook and Wilson, 1984) or to an increase in the concentration of other aromatic compounds such as amino acids.

The k' and Rs values were fairly reproducible but, as for most analyses using HPLC, these are likely to vary depending on the column used for the analysis and, unless the equipment is thermostated, on the ambient conditions (Anonymous, 1981; Goldberg, 1982). The order of elution of the mimosine and 3,4-DHP peaks in this study are identical to those reported for analysis by other HPLC techniques (Tangendjaja and Wills, 1980; Lowry, Tangendjaja and Cook, 1985).

Samples of LLM, LS and excreta extracted with 6M HCl and analysed using IEC (Acamovic and D'Mello, 1981) and HPLC are well correlated over about a 70 fold concentration range. (Table 3.1.1). Based on earlier work these results are likely to be higher than would be obtained if the samples were hydrolysed or extracted with charcoal (Acamovic and D'Mello, 1981). Extraction with 0.1M HCl without charcoal would be expected to give similar results to those obtained in this study but analysis using Fe(III) is likely to give much higher values. The use of HPLC has eliminated the requirement for treatment with charcoal and thus eliminates the inaccuracy associated with such a treatment (Matsumoto and Sherman, 1951; Megarrity, 1978; Acamovic and D'Mello, 1981, Lowry, Tangendjaja and Cook, 1985). The precision, and good agreement with IEC results, for the mimosine content of the LLM sample analysed at 269nm and 278nm assist in confirming the absence of interfering compounds.

The good recovery of supplemental 3,4-DHP to LLM and excreta is in accord with that of other workers using HPLC (Lowry, Tangendjaja and Cook, 1985) and paper chromatography (Hegarty, Court and Thorne, 1964). Quantitative recoveries indicate that there is no, or minimal interference from other compounds thus supporting the information obtained by calculating the ratio between peak areas.

The apparent absence of mimosine and 3,4-DHP in serum of chicks fed LLM, or perhaps the difficulty involved in detecting these in this study, is somewhat surprising since ingested mimosine in sheep produced plasma concentrations of up to about 0.4mM (Reis, Tunks and Hegarty, 1975). This is the only report which gives quantitative evidence of the presence of ingested mimosine in the plasma of animals while there are no reported levels of 3,4-DHP. Mimosine, intravenously infused into sheep caused detectable levels of up to about 0.5mM in the plasma (Reis, Tunks and Chapman, 1975). One report has demonstrated the presence of mimosine and, after considerable sample cleanup, 3,4-DHP in the serum of sheep fed *leucaena* (Tangendjaja and Wills, 1983) but no quantitative data were presented.

The absence of detectable mimosine and 3,4-DHP in serum of chicks fed LLM or LS and their presence in excreta is in direct contrast with chicks fed JB, where canavanine but not canaline was detected in serum but neither was detected in excreta. The presence of mimosine and 3,4-DHP in excreta, conforms with the numerous reports previously discussed (Chapter 1).

The lack of information regarding the presence of 3,4-DHP in serum, plasma and blood may be due to the hitherto unavailability of a suitably sensitive method for its analysis. This argument is not entirely correct for mimosine since two IEC methods have been developed for this purpose (Reis Tunks and Chapman, 1975; Mzik, 1977). One reason for the deficit of literary evidence for the presence of mimosine and or 3,4-DHP in blood may be the observation, here, that considerable losses of supplemental quantities of these in serum occur during protein precipitation and therefore if they are present in small quantities they may be completely lost during precipitation. (Table 3.1.3). Values reported for serum mimosine content of sheep infused with mimosine are likely to be under-estimates compounded by the fact that external standardisation was used (Reis, Tunks and Chapman, 1975). Use of an internal standard, however does not always give the correct results (Turnell and Cooper, personal communication; De Leenheer and Nellis, 1981; Hart and White, 1986).

It is well documented that protein precipitation from physiological fluids causes major difficulties in the quantitation of normal physiological amino acids (Williams, Hewitt, Cockburn Harris, Moore and Davis, 1980; Gardner, 1981; Williams, 1986) but is also apparently frequently neglected (Tews and Harper, 1985; Tews and Harper, 1986b; Demigne, Remesy and Fafournoux, 1986) or ambiguously reported with few supportive data (Turnell and Cooper, 1982).

The addition of mimosine to serum after the precipitation of protein (Mzik, 1977) gives excellent recovery of mimosine but does not give any indication as to losses incurred during precipitation. Recovery of supplemental mimosine and 3,4-DHP added to goat serum after protein precipitation is also reported to give good recovery of these (Tangendjaja and Wills, 1983) but similarly, does not provide information about the true value in untreated blood, serum or plasma. Perhaps hydrolysis could overcome the complications associated with the measurement of uncommon amino acids in proteinaceous matrixes.

Supplemental mimosine and 3,4-DHP in chick serum (Table 3.1.3) in this study showed that there were considerable losses of both during protein precipitation. Recovery was also dependent on the precipitant used and on the concentration of the analytes. Poor recovery is partially due to the losses caused in the

interaction of the analytes with the precipitant while adsorption to the precipitated protein may also be a contributing factor. Interestingly and in contrast, good recovery was obtained for supplemental canavanine when added to serum prior to precipitation with acetonitrile (section 3.2). The apparent absence of mimosine and 3,4-DHP in serum may be due to the conjugation of these compounds with other serum components such as carbohydrates to produce less toxic compounds such as the glucuronide (Tangendjaja and Wills, 1980; Reichert, 1981; Elliott, Norton, Milton and Ford, 1985).

4.1.2 Detection of mimosine and 3-hydroxy-4(1H)-pyridone using an electrochemical detector.

The good linearity of response to both mimosine and 3,4-DHP combined with the low detection levels with which this technique is endowed, give it high potential as 'the' HPLC technique for the estimation of mimosine and 3,4-DHP and their isomers and metabolites. It is the most sensitive and specific of all the chromatographic techniques available for the quantifification of mimosine and 3,4-DHP in biological matrixes.

Further work is however, necessary in order that the technique can be used for the analysis of these compounds in complex matrixes such as serum or animal tissues. The use of this, and other HPLC techniques may assist in elucidating the relationship between ingested, or infused, mimosine and 3,4-DHP and their metabolites, and their analogous physiological amino acids and amines.

4.2 Analysis of canavanine and canaline.

The use of HPLC for the determination of both canavanine and canaline has hitherto not been reported. From the work presented here it is obvious that HPLC is a potentially suitable technique for the estimation of these analytes simultaneously from a variety of matrixes, after OPA derivatisation. Chromatography using the larger column packed with Sherisorb ODS2 (dp=5um) produced good chromatography (Fig. 3.2.1a) with resolution of both canavanine and canaline, although the analysis time for canaline is fairly lengthy. The absence of canaline in all samples tested is, perhaps a reflection of lack of sensitivity of the method since canaline has been detected in JB during germination (Rosenthal, 1970; 1972). Modification of the sample preparation procedure may be necessary to detect and quantify canaline in JB and in other matrixes such as excreta and serum.

The detection and quantitation of canavanine were readily achieved using either the 160mm column with Spherisorb packing material or the shorter (100mm) column packed with ODS Hypersil (dp=3um; Fig. 3.2.1). In common with other HPLC analysis of amino acids and amines using OPA (Mell Jr., Dasler and Gustafson, 1978; Jones, Paabo and Stein, 1981; Turnell and Cooper, 1982) linearity of response was achieved. Similarly, reaction of canavanine with OPA and ethanethiol to produce the corresponding derivatives has also produced linear responses after quantitation using HPLC (Weissberger and Armstrong, 1984). The other methods available for the determination of canavanine also give linear responses (Rosenthal, 1977; Natelson and Bratton, 1984) but not always (Fearon and Bell, 1955).

Elution of canavanine, in the study reported here, using either column was possible under isocratic conditions, gave elution times of less than 8 min. Elution time for canavanine using the OPA/ethanethiol derivative was about 16 min using a gradient which was programmed for 40 min. The turnaround time using such a method could not, therefore, be less than 40 min. Peak shape and baseline stability using the ethanethiol derivatives appeared to be relatively poor.

Using the method developed here turnaround time can be as rapid as 15 min (Fig. 3.2.1b) for the analysis of canavanine if canaline is not present or ignored. A similar eluent gradient to that used for the shorter column for rapid analysis of canavanine can also be used for the Spherisorb column if canaline is ignored.

The chromatogram obtained from the extract of JB (Fig 3.2.2) has good baseline and peak characteristics in contrast to those obtained for extracts of lucerne tablets (Weissberger and Armstrong, 1984). The presence of the artifact peak from the OPA reagent was surprising since other workers (Turnell and Cooper, 1982) had reported that the reagent was indefinitely stable. The occurrence of the artifact peak is, however in agreement with other more recently reported work (Cooper, Ogden, McIntosh, Turnell, 1984). The presence of the artifact peak in the determination of canavanine and canaline is not significant, however it may interfere in the analysis of other amino acids in physiological fluids or hydrolysates.

The determination of canavanine in serum of chicks fed JB, using the Spherisorb column, was unsuccessful due to interference as reflected in the recovery of added canavanine although canaline was quantitatively recovered. The poor recovery of canavanine was surprising because the chromatogram of serum from chicks fed a JB free diet (Fig. 3.2.2b) showed a relatively clean baseline, and addition of canavanine to the canavanine free serum gave

quantitative recoveries. The use of the Hypersil column in resolving the interference however permitted slightly faster analysis and allowed quantitative recoveries of canavanine added to serum from chicks fed a diet containing JB.

The complete inability to detect canavanine or canaline in the excreta was interesting since it was expected that these amino acids would behave in a similar fashion to mimosine and 3,4-DHP. This inability to detect free canavanine or canaline in the excreta means that the concentration was considerably less than 250mg kg^{-1} DM. If canavanine had passed through chicks unchanged the approximate concentration would have been about 7g kg^{-1} DM.

It is possible that canavanine in JB, is more readily metabolised, or more available for metabolism, in comparison to mimosine in LLM. Canavanine and canaline may exist in a conjugated form in the excreta and therefore would not be amenable to detection by this method.

The excellent agreement for the values obtained colorimetrically and by HPLC, for canavanine in the water extract of a sample of JB (Table 3.2.1) is worth noting. Comparison of other techniques with the PCAF colorimetric method show a discrepancy between methods (Natelson and Bratton, 1984). This has been attributed to losses incurred in the often recommended use of charcoal to decolourise the extract of plant material (Fearon and Bell, 1955; Rosenthal, 1973). The extract procedures used by other workers are usually more complex than that described here and are likely to abstract more pigmenting components from the plant material (Natelson and Bratton, 1984; Natelson, 1985a; Weissberger and Armstrong, 1985). Perhaps the more exhaustive extraction procedures are necessary due to the complex nature of the matrix of some of the plant materials in which canavanine has been determined (Fearon and Bell, 1955; Bell, 1960). The adsorption of canavanine to charcoal, which is in agreement with the observations for mimosine, has been also been shown to be pH dependant and to depend on the type of charcoal used (Natelson and Bratton, 1984).

The good and reasonably precise recovery of canavanine, measured using both colorimetric and HPLC techniques (Table 3.2.2), may be due to the simple sample preparation technique used in the work reported here. This hypothesis is strengthened by the similarly good and precise recoveries of canaline from JB and excreta. The sample preparation technique used here may not be universally applicable however. The recovery of canavanine, by both techniques, is superior to that reported by others using either PCAF or PQ to permit detection (Fearon and Bell, 1955; Rosenthal, 1973; Natelson and Bratton, 1984).

The results obtained for canavanine in JB, and recovery of supplemental canavanine, indicate minimal interference. Confirmation of minimal interference is given by comparison of the canavanine concentration obtained by analysis of the hydrolysate using HPLC and IEC. Although the results are slightly lower (Table 3.2.1) the losses may be attributed to losses during hydrolysis in a similar manner to that for mimosine. The losses are somewhat contradictory to other reports where canavanine has been reported to be stable in acid solution (Rosenthal, Downum and Mattler, 1983; Natelson and Bratton, 1984). These observations, however, were made where canavanine only, was dissolved in pure solution whereas the results obtained during this work were with samples which included carbohydrate which is well known to assist in the degradation of amino acids (Ambler, 1981). The close correlation between IEC and HPLC results indicate that either technique can be used to give adequate results but the HPLC technique is more precise and much quicker. The use of hydrolysis to determine canavanine by either technique must be subject to question as with mimosine. The losses incurred here represent about 2g kg^{-1} of canavinine in JB. Samples containing levels approaching about 2g kg^{-1} may be reported as having no canavanine because all the canavanine has been destroyed during the hydrolysis procedure.

It is interesting to note that a canavanine standard solution which remained at room temperature for about 1 year produced two peaks on analysis by HPLC. Canavanine was the major peak but the later eluting one may be attributable to deaminocanavanine which is reported to be formed in aqueous solution at 30°C (Bell, 1960). This premise has not however been tested.

The HPLC methods developed during the course of this work, for the analysis of canavanine and canaline are not significantly hindered by sample pH and can be conducted routinely and rapidly with reasonably high precision. Further modification of sample preparation techniques may yield information on the presence or absence of canavanine and canaline in chick excreta, and canaline in serum.

It may be that canaline is present in serum but that it is present as a complex with pyridoxal phosphate (Rahiala, Kekomaki, Janne, Raina and Raiha, 1971; Rahiala, 1973) and this complex requires to be destroyed to release the canaline. The complexation of pyridoxal phosphate may account for the variation in serum ALT and AST activities (Tables 3.3.26 & 3.3.29) of chicks fed diets containing canavanine. Other conjugated forms of canavanine and canaline may

also be present in serum and excreta making detection impossible until the canavanine and canaline are deconjugated.

The technique described here is more sensitive and specific than paper chromatography with PCAF detection and may be useful in enhancing the taxonomic studies which have been conducted (Bell, 1958; 1960; Birdsong Alston and Turner, 1960; Turner and Harborne, 1967; Bell, Lackey and Polhill, 1978). During the course of the work reported here, lupin seed, which is in the family Papilionoideae, was extracted in a similar fashion to JB, and the extract tested for canavanine by HPLC. A peak with the same retention time as canavanine was detected and was equivalent to 0.17g canavanine kg^{-1} seed. When the standard canavanine solution was mixed with the lupin extract both peaks co-eluted thus supporting the proposition that the peak is due to canavanine. Further work is required to confirm this finding.

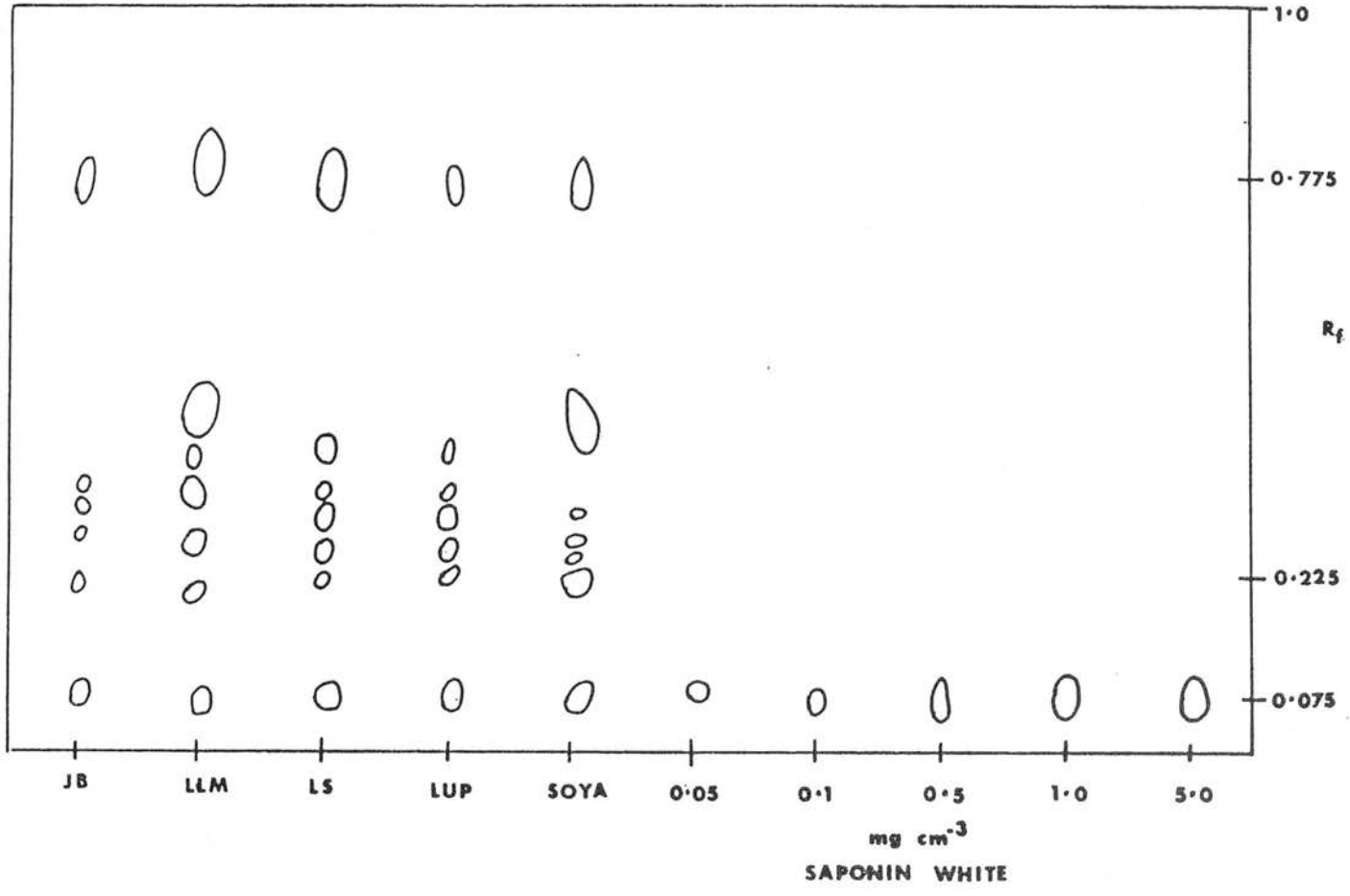
Canavanine has been reported not to occur in *Lupinus* species (Bell, Lackey and Polhill, 1978) but the presence of canavanine at the level reported here would not be detectable using the PCAF method which has been used frequently to screen for the presence of canavanine in plant material (Bell, 1958; 1960; Birdsong, Alston and Turner, 1960; Turner and Harborne, 1967). The amount of canavanine present in the sample taken for analysis by these workers would approximate 0.2ug which is far lower than that required to produce a detectable colour with PCAF (Bell, 1958; 1960; Natelson and Bratton, 1984). The presence of canavanine at this level in *Lupinus* albus may have phytochemical and taxonomic significance but little significance nutritionally as will be discussed later.

The polemics regarding the suitability of PCAF for the estimation of canavanine (Rosenthal and Dahlman, 1982; Natelson, 1985) are unlikely to occur with respect to the HPLC method described here.

4.3 Qualitative estimation of saponins by thin layer chromatography.

Saponins were detected visually from the TLC plates after chromatography. This is obviously a relatively imprecise and inaccurate method for the estimation of any compound separated by this technique. Quantitation is made more difficult in this case, by the complexity of the analytes and the difficulty incurred in spraying the plates evenly. Accepting the limitations of the technique it is possible to qualitatively assess the presence of saponins and to make an approximate assessment of quantity by comparison with standards.

Fig. 4.3.1 Representation of a TLC plate of 10 µl extracts of jack beans (JB), *Leucaena* leaf meal (LLM), *Leucaena* seeds ((LS), lupin seeds (LUP), soya beans and saponin white solutions.



The TL chromatogram of the extracts of JB, LLM, LS, lupin seed, soya bean and saponin white (Fig. 4.3.1) show clearly the presence in all the samples, of a component with the same Rf value (0.075) as saponin white. It is also notable that there are several spots from soya which have an Rf values which are common to those spots from the other samples.

It is obvious from the recent literature on the estimation of saponins in plant material, that TLC is not an accurate method for the estimation of these compounds; yielding values which are inflated. However, the more sophisticated methods described in the recent literature are not trouble free and are also somewhat inaccurate (Ireland and Dziedzic, 1985; Price, Curl and Fenwick, 1986; Ireland and Dziedzic, 1986). If the sapogenin content is accurately determined the saponin content of the plant material is still a guesstimate because of the unknown degree of conjugation with carbohydrates and the unknown nature of some of the saponins (Ireland and Dziedzic, 1985; Price, Curl and Fenwick, 1986; Ireland and Dziedzic, 1986; Ireland, Dziedzic and Kearsley, 1986). Analysis of the extracted saponins by HPLC shows most promise in accurately and precisely quantifying their content in plants but standards are required (Burnouf-Radosevich and Delfel, 1986).

The results presented in this study show that saponins, or saponin like compounds, are present in LLM, LS, lupin seed and JB and the concentrations are in the range of about 2-10g kg^{-1}. Hitherto, saponins have not been reported to occur in LLM, LS or JB although they have been reported in lupin seed as determined using TLC with haemolytic detection (Hudson and El-Difrawi, 1979; Haraszti and Vetter, 1983; Muzquiz, Vidal and Cassinello, 1984). Haemolytic detection utilises a general property which is specific to certain types of saponins (Applebaum, Marco and Birk, 1969; Curl, Price and Fenwick, 1985) which may or may not be present in LLM, JB or LS. If, in any previous work, this method was used to test for saponins in these samples, they may not have been detected.

Recent work (Price, Curl and Fenwick, 1986) in which fifteen different legume seeds were assayed for saponins, showed that analysis by TLC produced saponin spots for all samples under study including JB. In that study however, determination of the saponins as their aglycones (sapogenins) by gas chromatography (GC), only the extract from JB did not yield peaks. The discrepancy between the TLC and the GC analyses for JB is perhaps partially explained if the saponins present in JB do not produce soyasapogenols B and C (Price, Fenwick and Jurzysta, 1986) which were used as the standards. Soyasapogenols B and C were produced by hydrolysis of soyasaponin I (Fig.

1.8.5.1). It was not clear whether other peaks were detected (Kitagawa, Yoshikawa, Hayashi and Taniyama, 1984a; 1984b; Ireland and Dziedzic, 1985; 1986; Ireland, Dziedzic and Kearsley, 1986; Price, Fenwick and Jurzysta, 1986) and if so, whether or not these were taken into account.

It is evident that the values for saponin content of the samples used here are likely to be high because of the method of estimation. However it appears that all the samples used in the work reported here, do contain saponins but some controversy exists regarding their presence in JB as well as their content and variety in these and other plant materials.

Further work using more specific methods is necessary in order to identify and quantify accurately, the saponins present in the legumes used in this study.

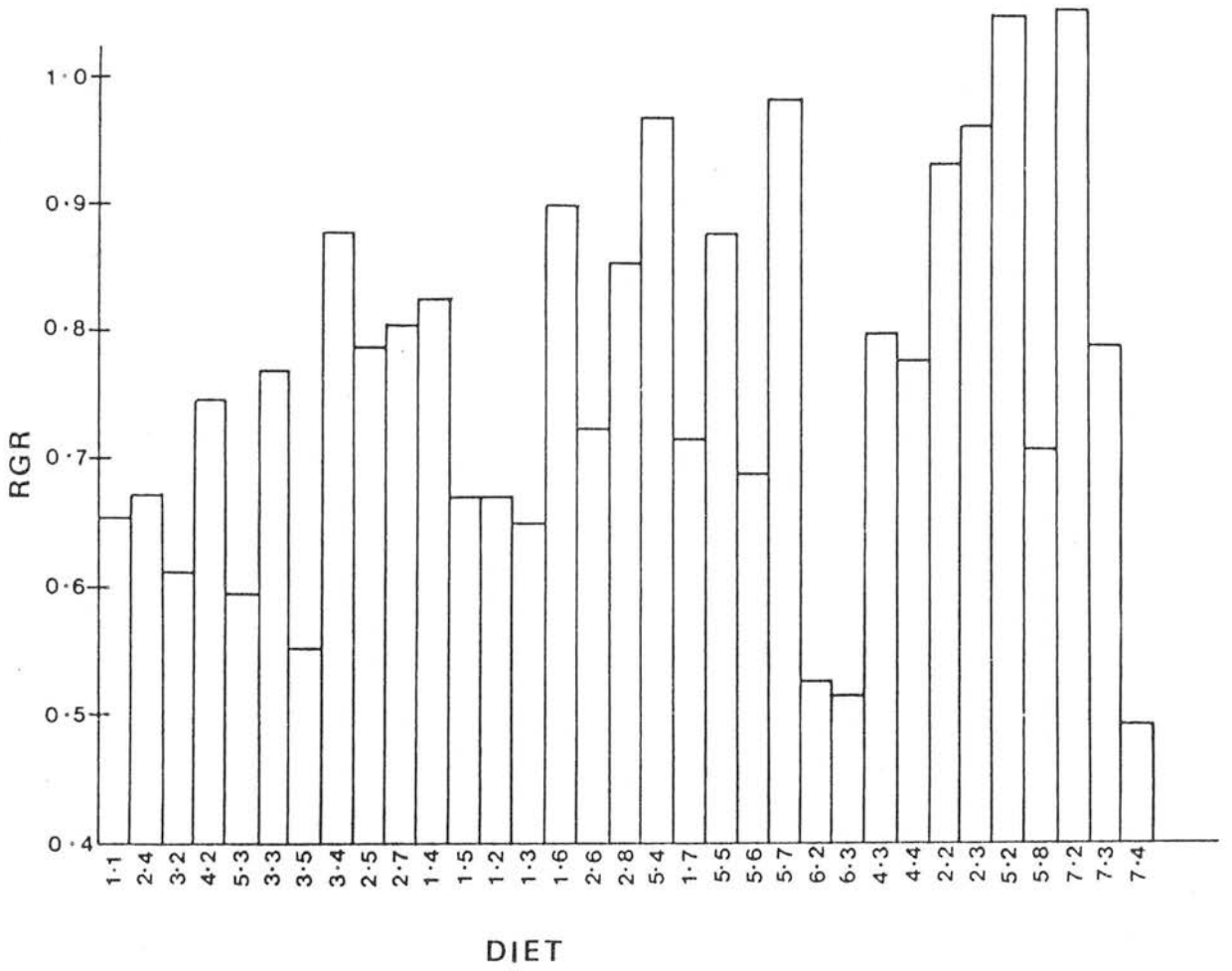
4.4 Chick experiments

4.4.1 Experiment 1.

The inclusion of LLM in chick diets is widely reported to be deleterious to growth (Springhall and Ross, 1965; Labadan, 1969; D'Mello and Thomas, 1978; D'Mello and Taplin, 1978; D'Mello and Acamovic, 1982). One report has, however, shown that dietary inclusion of a particular batch of LLM from Malawi, at 150g kg^{-1} did not induce a growth depression (D'Mello and Acamovic, 1982). That batch of LLM had lower fibre, tannin and mimosine contents than others (D'Mello and Fraser, 1981; D'Mello and Acamovic, 1982). Responses of chicks to the inclusion of LLM at 150g kg^{-1} in the experiments reported here are in agreement with previous findings, particularly for unsupplemented diets. The relative growth rates (RGR) of chicks (ie growth rate of chicks fed any diet/growth rates of chicks fed the control diet) fed diets containing LLM, irrespective of treatment, were all lower than control fed birds (Fig. 4.4.1).

The use of PEG4000, PVP and other polymers in attempts to reduce the antinutritional and growth depressing effects of sorghum, which have been attributed to tannins, have shown some success (Armstrong, Featherston and Rogler, 1973; Savage, Smith and Briggs, 1978; Hewitt and Ford, 1982; Deshpande, Sathe and Salunkhe, 1984). In contrast to the work on sorghum, the inclusion of PEG4000 into diets containing 150 g LLM kg^{-1} diet, at 20 or 40g kg^{-1} in experiment 1, had no effect on growth and food intake and little effect on EFC for animals fed these diets (diets 1.2 & 1.3). The lack of response in this case, is likely to be due to the more dominant effects of other components,

Fig. 4.4.1 Relative growth rates (RGR) of chicks fed diets containing Leucaena leaf, Leucaena seed, mimosine and sorghum



RGR = Growth Rate

Growth rate of chicks fed the control diet for each experiment

such as mimosine and other phenolic compounds including quercitin, found in *leucaena* (Lowry, Cook and Wilson, 1984). Soyabean/maize meal diets supplemented with PEG4000 have also been shown (D'Mello, unpublished work) to have no effect on chick growth; in that case it was probably due to the absence of significant quantities of polyphenolic compounds.

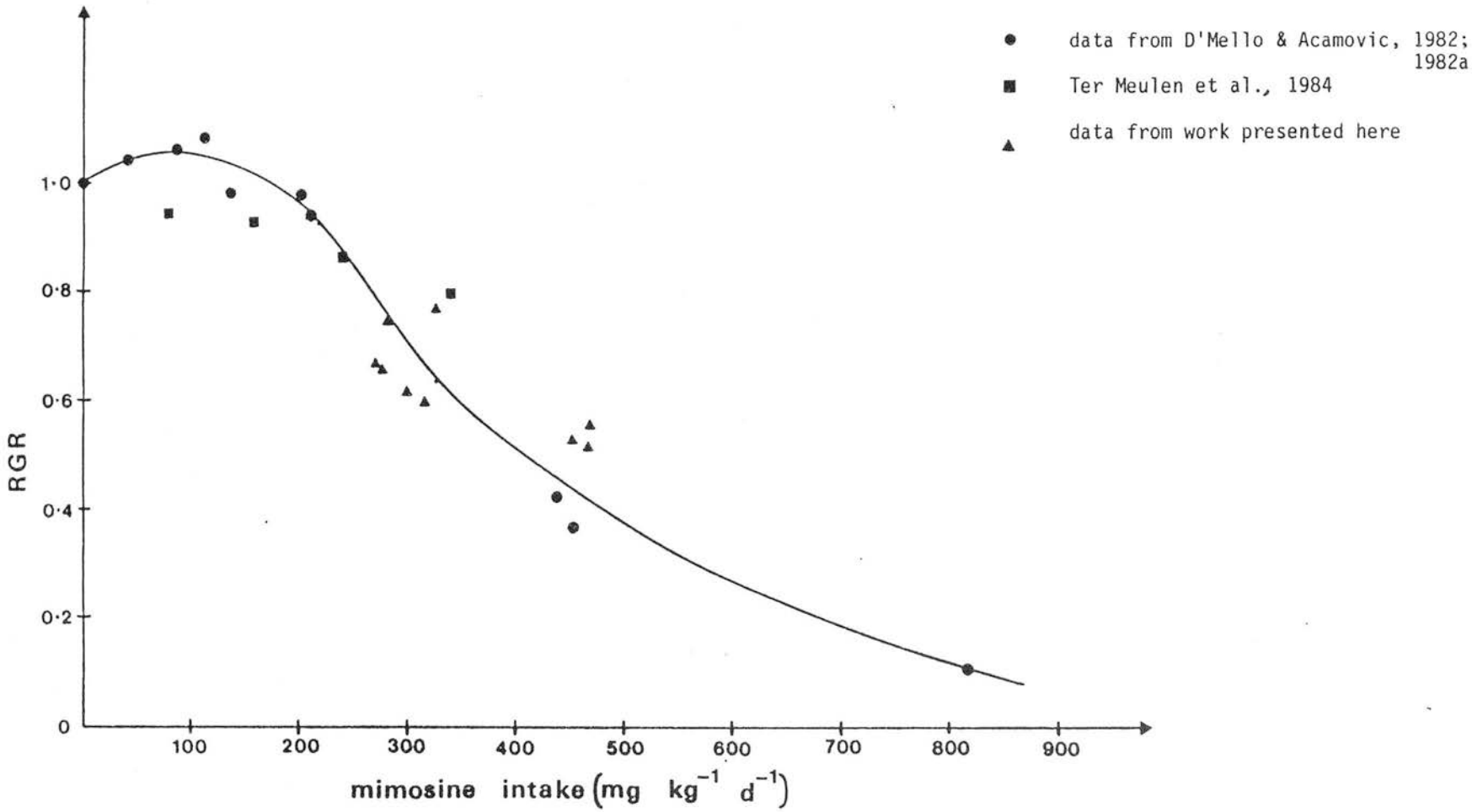
The increase in growth rate, with concomitant increase in food intake (Table 3.3.1), which resulted from the addition of $Fe_2(SO_4)_3$ at 12g kg^{-1} (diet 1.4) is in accord with responses to supplemental Fe(II) (Table 4.4.1; Ross and Springhall, 1963). The lack of effect of adding $Fe_2(SO_4)_3$ at 24g kg^{-1} (diets 1.5 & 1.7) on growth may be a result of a reduction in dietary pH (Table 2.13.1).

Dietary pH has been shown to adversely affect growth rate and EFC in chicks (Pritzl and Kienholtz, 1973). So, in experiments in which Fe(III) or Fe(II) is added to the diets to complex with phenols, two opposing effects may occur; the chelation effect may enhance performance while the pH effect is deleterious to performance. This trend is observed in experiments 2 and 7 where supplementation of diets, which produced good growth rates (Tables 3.3.6 & 3.3.21; Fig. 4.4.1), with Fe(III); reduced dietary pH (Table 2.13.3) and also caused a decrease in growth. This may also account for the the fact that LLM diets supplemented with $Fe_2(SO_4)_3$ at levels between 1.6 and 12.0g kg^{-1} elicited almost equivalent RGR values which were higher than those for unsupplemented LLM diets, while those fed the diet with 24g kg^{-1} $Fe_2(SO_4)_3$ had a considerably lower RGR (Fig. 4.4.1).

The inability of Fe(III) supplemented LLM diets to produce growth equivalent to those birds fed control diets (Fig. 4.4.1) may be partly due to the dichotomy between the deleterious effects of pH and the beneficial effects of chelation of the phenolic compounds including mimosine, with Fe(III). Chelation of Fe(III) and phenols is most effective at low pH (Tsai and Ling, 1973). The presence of other antinutritional factors such as saponins, enzyme inhibitors and complex carbohydrates must not, however be overlooked and are likely to contribute to the decrease in RGR.

In experiment 1 addition of PEG4000, in combination with with Fe(III) had a beneficial effect on growth, food intake and EFC in comparison with their respective PEG4000 free diets (Table 3.3.1; Fig.4.4.1). The implication therefore is that PEG4000 reduces the adverse effects associated with the presence of polyphenolic compounds with relatively high RAM (sect 1.8.4) while Fe enhances the nutritional value by interaction with both small and large polyphenolics (Wah, Sharma and Jackson, 1977; Rao and Prabhavathi, 1982).

Fig. 4.4.2 Plot of mimosine intake (mg^{-1} liveweight d^{-1}) vs. relative growth rate (RGR) of chicks fed various diets containing *Leucaena* leaf meal.



Addition of Fe(III) to the diet permits a higher relative intake of mimosine and DHP in chicks (Table 3.3.4) without the same consequential loss in performance in birds fed LLM diets without supplemental Fe(III) (Fig. 4.4.2). It is interesting to observe that chicks fed LLM diets with differing mimosine contents show that growth depression occurs when normalised mimosine intakes exceed about 175mg kg^{-1} liveweight (Fig. 4.4.2). This is remarkably close to the value quoted by other workers (ter Muelen, Pucher, Szyszka and El Harith, 1984). The growth depression is most severe in this experiment where mimosine and 3,4-DHP is not adequately accounted for in the excreta (Table 3.3.5). However complete excretion of mimosine and 3,4-DHP did not permit birds to perform at the same level as control fed birds.

The ratio of mimosine excreted to that consumed for birds fed the unsupplemented LLM diet is similar to that reported elsewhere for chicks (D'Mello and Acamovic, 1982). Interestingly the loss of mimosine is not accounted for as 3,4-DHP (Table 3.3.5) in the excreta and may be lost as their conjugates as has been reported for other animals (Tangendjaja and Wills, 1980). The loss of mimosine in chicks fed the LLM diet supplemented with 24g kg^{-1} $Fe_2(SO_4)_3$ however, is essentially accounted for as 3,4-DHP. This may be due to the reduced pH associated with the high content of Fe(III) which would tend to assist the hydrolytic cleavage of mimosine either enzymically or chemically within the animal (Tangendjaja, Lowry and Wills, 1984).

Variation in carcass composition in poultry is associated with nutrient intake and the ratio of energy to protein in the diet (Sibbald and Wolynetz, 1985a; 1985b; Mbugua, Austic and Cunningham, 1985; Jones, 1986). The carcass component which varies most readily by variation in intake and dietary energy to protein ratio, is fat (Sibbald and Wolynetz, 1985a; 1985b; Mbugua, Austic and Cunningham, 1985; Jones, 1986). Since the dietary energy to protein ratios in this experiment are essentially the same the variation in carcass composition and efficiency of deposition of nutrients can be attributed to, and in general reflects, food intake (Tables 3.3.1, 3.3.2, 3.3.3). The reduction in food intake may be attributable to the intake or presence of free mimosine and its metabolites in the absence of supplementary Fe(III), which may affect the food regulating mechanism within the animal. This aspect of mimosine on food regulation will be discussed later.

4.4.2 Experiment 2.

As mentioned in the previous section, growth and food intake for chicks fed the soya/maize diets with or without supplemental Fe (diets 2.1, 2.2, 2.3) were higher than for diets containing LLM (Table 3.3.6). Supplemental Fe adversely influenced both growth rate and food slightly which is in agreement with effects of reduced dietary pH (Pritzl and Kienholtz, 1973). Relative growth rates of chicks fed the Fe supplemented soya/maize diets were also lower than some of those fed the LLM diets (Fig. 4.4.1). In this experiment, however, addition of PEG4000 and $Fe_2(SO_4)_3$ at 20 and 1.6g kg^{-1}, respectively, to an LLM diet (diet 2.6) produced a lower growth rate than the equivalent diet (diet 2.5) without PEG4000 but food intake was not affected. The PEG supplemented diet produced a lower RGR (Fig. 4.4.1) than all the Fe supplemented LLM diets except for that supplemented with 24g $Fe_2(SO_4)_3$ kg^{-1} diet (1.8). In contrast, growth was similar for the LLM diets with supplemental $Fe_2(SO_4)_3$ at 4.8g kg^{-1} diet where PEG4000 had no effect.

The growth depression and accompanying reduction in EFC caused by PEG4000 addition to the LLM diet supplemented with 1.6g $Fe_2(SO_4)_3$ is not readily explained but is apparently related to the ratio of mimosine and other phenolic compounds to Fe(III) in the presence of PEG4000. Other diets containing both PEG4000 and Fe(III) did not adversely affect RGR values (Fig. 4.4.1) of chicks fed these diets. The ratio of Fe(III) to mimosine in the diet did not, however, affect the mimosine and 3,4-DHP output compared with chicks fed the other LLM diets (Table 3.3.10). The ratio of mimosine excreted to that consumed, for diets supplemented with Fe(III), was in accord with the results for experiment 1.

The essentially quantitative excretion of mimosine from chicks fed dietary LLM without supplemental Fe(III) is contrary to the results obtained in experiments 1, 3 and 4 as well as results reported elsewhere (D'Mello and Acamovic, 1982). This phenomenon cannot be accounted for by higher levels of Fe in the diet since these are essentially the same in experiments 1, 2 and 3 (Tables 2.13.1, 2.13.3 & 2.13.4). It is not possible to satisfactorily account for this discrepancy but it may be related to the ability of the gut microflora to degrade mimosine as has been shown for ruminants (Jones and Megarrity, 1983).

The higher ratio of excretion of mimosine to intake may, however, be reflected in the slightly better RGR for chicks in this experiment than those in experiments 1, 3 and 5 but not 4 (Fig 4.4.1). The losses of 3,4-DHP may be due to effective conjugation or degradation of the 3,4-DHP within the digestive tract

of the chicks. The resultant products would not be quantified using the HPLC methods developed during the course of this work since chromatographic retention of the conjugates are likely to be greater than the unconjugated compounds (Tangendjaja and Wills, 1980).

As discussed earlier (sect. 4.4.1) carcass composition reflects food intake and this can adequately account for the higher fat content associated with the birds fed the LLM free diets (diets 2.1; 2.2; 2.3). These birds had a higher food consumption than birds fed LLM diets (Tables 3.3.6 & 3.3.7). The higher lipid content of the chicks is reflected in the gross energy values. The carcass composition and efficiency of deposition of nutrients of the LLM fed birds were similar to those in experiment 1. The effect of Fe supplementation which reduces the effects of mimosine by chelation, appeared to have little effect on carcass composition. By implication mimosine appears to have little direct effect on chick carcass composition.

4.4.3 Experiment 3.

The inclusion of LS at 65g kg^{-1} into chick diets produced a growth depression (Table 3.3.11) which was not unexpected due to the presence of tannins, mimosine, saponins, galactomannan gums, and haemagglutinins as well as potent trypsin inhibitors (Tables 2.8.1, 2.13.4; section 4.3; Lesniak and Liu, 1977; 1981b). Similar growth depressions have been observed by other workers (Lee and Yang, 1982) for chicks fed equinitrogenous diets. These workers found that growth was depressed although the seeds had been soaked in H_2O for 24h and boiled for 1h prior to incorporation of the dried material into the diets. Growth depressions, which were attributable to mimosine content of the diets, were also observed when LS was fed, as a mixture with LLM, to chicks (ter Meulen, Pucher, Szyszka and El-Harith, 1984). It seems reasonable that the trypsin inhibitors present in LS (Table 2.13.4) would have significantly affected performance. The RGR values obtained by these workers (ter Muelen, Pucher, Szyszka and El-Harith, 1984) are not strictly comparable with those reported here because the *leucaena* meal was substituted at the expense of lucerne meal.

It is well documented that lucerne meal, primarily due to its saponin content (sect. 1.8.5), depresses growth and food intake of chicks and other monogastric animals monogastric animals (Cheeke, 1976; Cheeke, Pedersen and England, 1978; Hegsted and Linkswiler, 1980; Ueda, Ohshima and Kamada, 1986). Thus the RGR of birds fed the *leucaena* meal in that experiment (ter Muelen, Pucher, Szyszka and El-Harith, 1984), are likely to be higher than in those reported here.

Notwithstanding this problem the RGR values when related to normalised mimosine intakes compare reasonably well with those reported here (Fig. 4.4.2).

An interesting observation in experiment 3 is that the diet with LS (diet 3.3), without Fe(III) supplementation, sustained better growth rates and food intakes than chicks fed an LLM based diet with the same mimosine content. In fact the RGR value was higher than that for chicks fed any of the basal LLM diets with the same mimosine content (Fig. 4.4.1) and as good as LLM diets with supplemental Fe. The better performance was obtained despite the presence of potent trypsin inhibitors in LS (Table 2.8.1)

The differences in RGR between LLM and LS fed birds, where dietary mimosine contents were the same, strongly suggests and supports the view that there are other major antinutrients present in LLM which influence good performance. The discrepancy in RGR may be partially attributable to the absence of detectable amounts of 3,4-DHP in the seeds while apreciable amounts of 3,4-DHP are present in LLM (Table 2.13.2). This may indicate that 3,4-DHP has an adverse effect on chick growth in a similar manner to that which has previously been demonstrated in mice (Hegarty, Lee, Christie, Court and Haydock, 1979). It has been assumed that 3,4-DHP was also the cause of depression of growth in chicks, ducks and rabbits fed *leucaena* leaf which had been heated prior to incorporation of the dried material into the diet (Tangendjaja and Lowry, 1984). The mimosine or 3,4-DHP contents of the treated *leucaena* were not reported by these workers.

The reduction in growth rate, food intake and EFC associated with chicks fed the diet with 95 g LS kg^{-1} diet (diet 3.5) is probably due to the increased concentrations of all the antinutritive factors in the diet. The effect of the trypsin inhibitors present in LS may be a major factor in producing these responses which are similar to those observed in rats fed diets with different trypsin inhibitor activities (Kakade, Hoffa and Liener, 1973; Grant, McKenzie, Watt, Stewart, Dorward and Pusztai, 1986).

Supplementary Fe(III) did not enhance the excretion of mimosine or 3,4-DHP from chicks fed LS which is at odds with experiments in which LLM diets supplemented with Fe(III) produced quantitative excretion of mimosine (D'Mello and Acamovic, 1982a).

Although Fe(III) supplementation had little effect on the proportion of mimosine and 3,4-DHP excreted the growth of chicks fed the different diets (diets 3.3, 3.4) were different. This result is in agreement with results obtained in experiments

1, 2 and 4. The implication is that mimosine, 3,4-DHP and other phenols cause a reduction in available Fe, and perhaps other metals by complexation, which is reversed by addition of Fe to the diet.

The lack of effect of Fe(III) supplementation on the proportion of mimosine and 3,4-DHP excreted may also be due to the slightly higher tannin content in the diet from LS combined with the slightly lower dietary Fe content (Table 2.13.4). If this was the case then it might be expected that the diet containing LS at 95g kg^{-1} (diet 3.5) would have had a lower mimosine excretion to intake ratio because the Fe to dietary phenolic content is lower for this diet than that for the Fe(III) suplemented diet (3.4).

The results reported here for the ratio of mimosine excreted to that consumed are in agreement with those in experiment 4 where Fe(II) suplementation did not induce quantitative excretion of mimosine. As postulated earlier, the cause of variation in the relative excretion of mimosine is not readily explained but may be due to variation in gut microflora activity.

The RGR of chicks fed the LS diet supplemented with Fe(III) (diet 3.4) was higher than any of the LLM diets supplemented with Fe (Fig. 4.4.1). It is of note that the food intake was essentially the same as those birds fed the control diet indicating, in this case, that food intake was not the cause of the reduced growth rate.

4.4.4 Experiment 4.

The improvement in growth rate on addition of Fe(II) to LLM diets (diets 4.3 & 4.4) is of the same order as chicks fed diets with equal quantities of LLM and Fe(III) (Fig. 4.4.1). This phenomenon is anomolous to the chemical data (Tsai and Ling, 1983) which indicates that the Fe(II) mimosine chelate is much weaker than the Fe(III) chelate although both Fe(II) and Fe(III), when added to tannin, have been shown to be effective at binding tannin (Wah, Sharma and Jackson, 1977; Rao and Prabhavathi, 1982). The similarity of response may be associated with the relative solubilities of Fe(II) and Fe(III) salts and their ability to be absorbed by the animal as well as the ability of Fe to change valency (Park, Mahoney and Hendricks, 1983) all of which may be influenced by the presence of phenolic compounds. The results obtained indicate that Fe(II) is as effective as Fe(III) at reducing the adverse effects of phenolics found in LLM.

4.4.5 Experiment 5.

A slight increase in growth rate and food intake was observed for the chicks fed the soya maize diet with supplemental PVP-40 (diet 5.2) compared with those fed the control diet (5.1). The difference may have been due simply to an increase in palatability of the former diet caused by the higher oil content of the diet (Table 2.13.6). Supplementation of other soyabean maize diets with PVP-40 has been shown to have no effect on growth or food intake (D'Mello, unpublished work).

The large improvement in growth rate (Table 3.3.15; Fig. 4.4.1) and food intake of chicks fed the LLM diet with supplemental Fe(III), PEG4000 and cholesterol (diet 5.4) is only superceded by chicks fed either control diets or with the similar diet containing heat pretreated LLM (diet 5.7). The difference between control fed birds and those fed the LLM diets is that for the same food intake, growth is slightly lower for LLM fed birds (Table 3.3.15) which indicates that LLM has toxic and/or antinutritional effects rather than its effect on food intake. The inclusion of PVP-40, Fe(III) and cholesterol in the LLM diet (diet 5.5) appears to have had a similar positive effect on growth and food intake as PEG4000 (Table 3.3.15 & Fig. 4.4.1). Quail fed PVP (RAM not given) and LLM in diets at the same levels as those described here, but without additional Fe(III), showed a slight enhancement of growth compared with birds fed the equivalent diet, but without PVP supplementation (Hegde, Ross and Brewbaker, 1983). In that experiment the fat content of the PVP supplemented diet was lower than its unsupplemented LLM reference diet thus supporting the theory that PVP enhanced growth by interference in the formation of protein-phenol complexes.

There are a number of possible reasons for the increased performance observed for birds fed treated LLM diets. Supplemental cholesterol, Fe(III) and PEG4000 was accompanied by a slightly higher oil content in diets 5.4 and 5.7 (Table 2.13.6) which may have enhanced the palatability of the diet. The beneficial response may also be partially attributed to the synergistic effects and "extracaloric effects" of the additional oil (Jensen, Schumaier and Latshaw, 1970; Horani and Sell, 1977; Owen, Waldroup, Mabray and Slagter, 1981; Wiseman, Cole, Perry, Vernon and Cooke, 1986). These effects on dietary AME are however, not observed in the experiments reported here (Table 3.3.17). The higher concentrations of oil, in combination with cholesterol, may enhance the production of micelles (Sidhu and Oakenfull, 1986; Oakenfull, 1986) associated with the presence of saponins in the diet which would enhance the excretion of lipid material (sect. 1.8.5). Losses of these compounds in the excreta would mask

any synergistic or "extra caloric" effects caused by extra dietary oil and would also minimise the effects of dietary saponins.

The improvement in growth rate, EFC and food intake of chicks fed diets in which the LLM was cooked prior to inclusion (diets 5.6 & 5.7) is in accord with work with rats and is indicative of the destruction of heat labile antinutritive factors such as trypsin inhibitors (Table 2.8.1) and a-amylase inhibitors (Jaffe, 1973; Grant, McKenzie, Watt, Stewart, Dorward and Pusztai, 1986). It is notable, however, that trypsin inhibition is not completely alleviated in the unsupplemented LLM while complete destruction was obtained when PEG4000 and Fe(III) was incorporated prior to cooking (Table 2.8.1). The implication is that there are heat stable trypsin inhibitors such as some polyphenolics, including mimosine (Fujiya and Tawata, 1985), present in LLM (sect. 1.8.4). Since PEG4000 has an affinity for tannins (see sect. 1.8.4 & earlier discussion), and Fe(III) for phenolics including tannins (Rao and Prabhavathi, 1982), addition of both of these prior to autoclaving is likely to have caused interaction with the polyphenolics thus reducing the adverse affects of these on the the digestive enzymes and therefore on the chicks (Griffiths, 1979; Marquardt and Ward, 1979; Griffiths and Moseley, 1980).

Cooking the LLM is also likely to have enhanced protein and carbohydrate availability and to have increased their respective susceptibility to digestion as has been reported for other legumes (Jaffe, 1975; Shannon and Clandinin, 1977; Marquardt and Ward, 1979; Reddy, Pierson, Sathe and Salunkhe, 1984). The effect of heat treatment on the carbohydrates in the LLM may be to reduce the 'sweep out' effect associated with complex carbohydrates (sect. 1.8.6) which is reflected in the higher ENR and EDE values (Table 3.3.18) for chicks fed the diets containing the heat treated LLM. The corollary to heat treatment, which should not be overlooked, is the formation of Maillard type compounds (sect. 1.8) which may partially account for the slightly poorer RGR for chicks fed the diet with supplemented and cooked LLM (diet 5.7) than for control fed birds (Fig.4.4.1). Autoclaving did not appear to enhance the AME of LLM contrary to the effects of cooking on the AME of other legumes (Shannon and Clandinnin, 1977; Molina, Sanz, Boza and Aguillera, 1983; Nwolokolo and Oji, 1985).

The reduction in growth associated with the addition of mimosine to a soya bean maize diet (diet 5.8) at a level equivalent to that for an LLM suplemented diet, was large compared with control fed birds (Table 3.3.15). The RGR value of birds fed the mimosine supplemented diet was higher than that for all but one of the values obtained for LLM basal fed chicks (Fig.4.4.1). The implication is that mimosine is the most potent antinutritive component in LLM but this

premise is not supported by the inability of Fe(III) or Fe(II) suplementation to achieve control fed bird responses. The response of chicks to PEG4000, cholesterol and heating also supports the view that antinutritive factors other than mimosine and 3,4-DHP are present in LLM.

It may be that mimosine added to the diet is more readily available than mimosine present in LLM and LS; some of which may be contained within the cellular material. The ease of extraction of mimosine and 3,4-DHP reported here and elsewhere (Hegarty, Court and Thorne, 1964; Acamovic and D'Mello, 1981; Lowry, Maryanto and Tangendjaja, 1983) would indicate that these compounds are reasonably available within the matrix of the plant material and therefore, also in the gastrointestinal tract. This, however, is in contradiction to the work reported here, and elsewhere (D'Mello and Acamovic, 1982) where most of the mimosine and 3,4-DHP ingested is excreted and there is no detectable quantity of mimosine or 3,4-DHP in the serum yet considerable growth depression, which is alleviated to some extent by the presence of Fe(III), occurs in animals fed LLM.

The argu ment could then be put that mimosine and 3,4-DHP have minimal effect on growth if consumed as an integral part of LLM and other components in LLM cause the growth depression. This hypothesis must be refuted because treatment of the diets to alleviate the effects of other harmful components has little or no effect (Fig.4.4.1). It is also possible to hypothesise that mimosine and/or 3,4-DHP is so potent that the very small amounts which may be absorbed from the gastrointestinal tract, cause a large effect in chicks. This does not agree with other work where mimosine excretion and low intake had no perceptible effect on chick growth while increased mimosine intake, albeit by increasing the amount of LS or LLM in the diet, caused growth depressions (D'Mello and Acamovic, 1982; ter Muelen, Pucher, Szyszka and El-Harith, 1984). Mimosine and/or 3,4-DHP ingestion in the experiments reported here may be so excessive that losses due to absorption in the digestive system would be negligible. The aproximately equal response of chicks to the range of concentrations of Fe(III) supplementation of LLM diets militates against this arguement however.

When food intake of chicks fed a control diet (diet 8.1) was restricted and maintained at the same level as chicks fed a diet with a similar quantity of mimosine (diet 8.2) to that in experiment 5 (diet 5.8), growth rates for the chicks fed the same quantity of diet were the same. Food intake in experiment 8 was restricted to about 0.857 of the ad lib food intake; this was higher than the ratio of food intake for birds fed the control and mimosine supplemented diets

Fig. 4.4.3 Biosynthesis of catechololamines

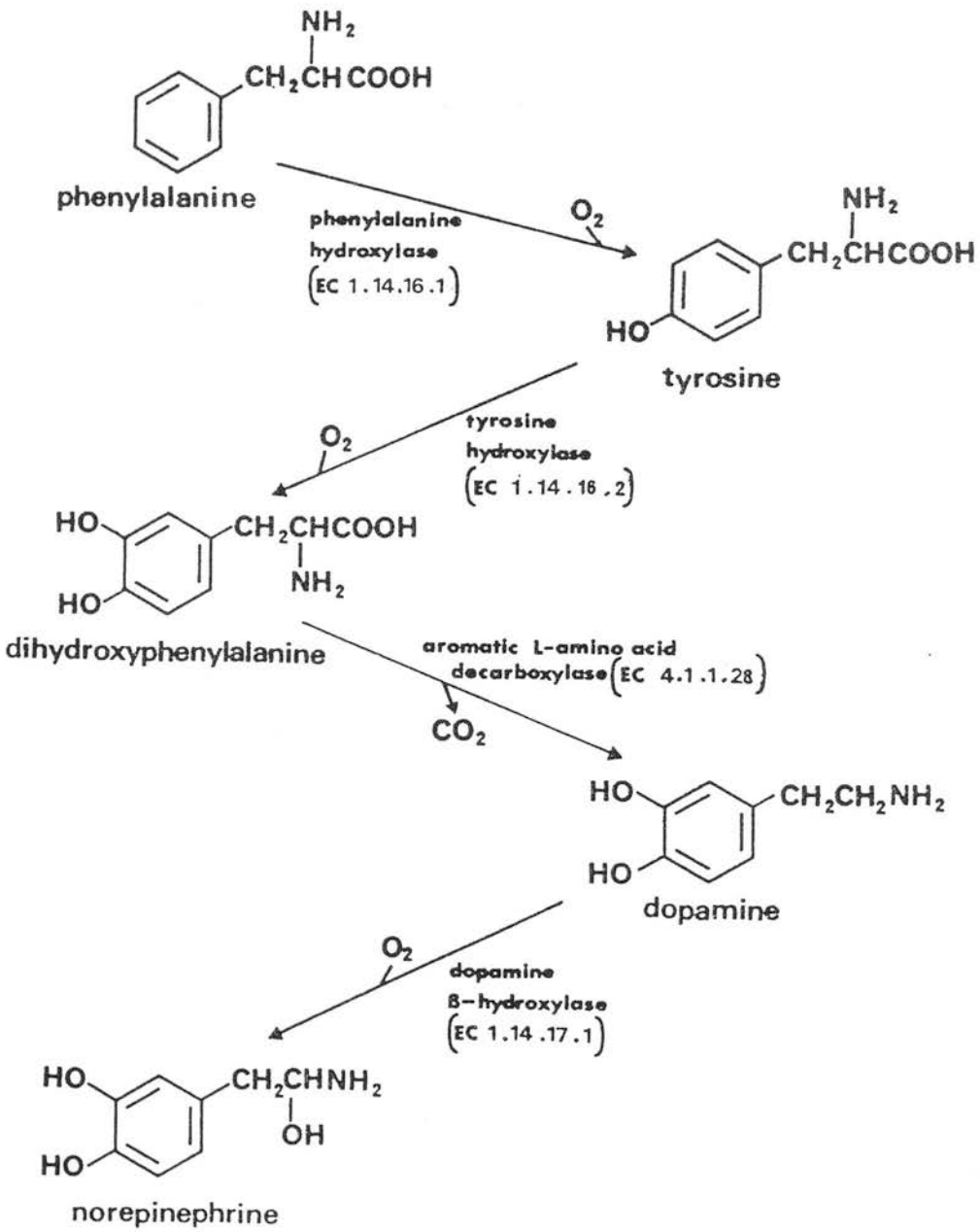
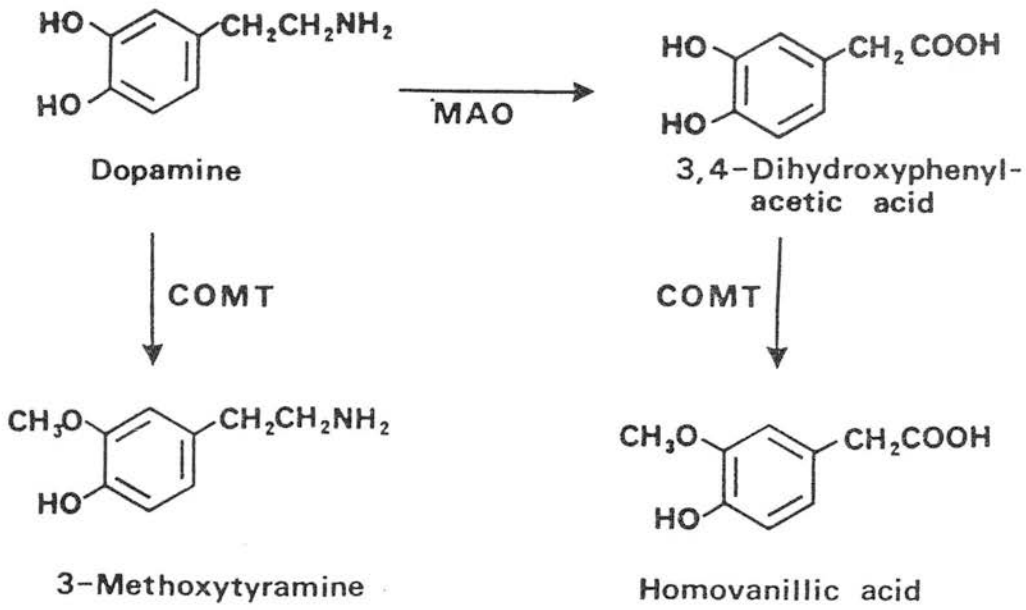


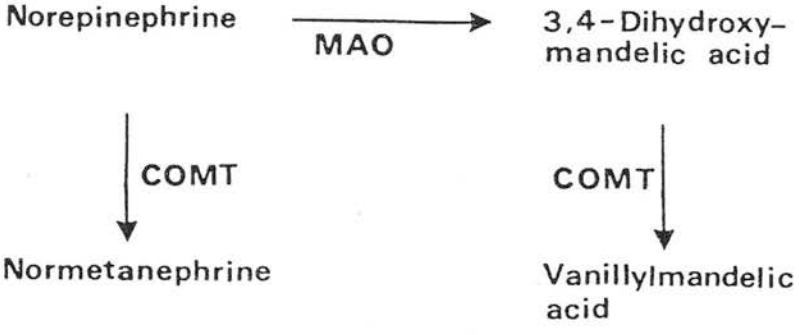
Fig. 4.4.4. Catecholamine catabolism



MAO = Monoamine oxidase (EC 1.4.3.4)

COMT=Catechol-o-methyl transferase(EC 2.1.1.6)

similarly;



(diets 5.1 & 5.8), on an ad lib basis, in experiment 5. The relative ad lib intakes of chicks fed the basal LLM diets in all the other experiments was lower than that for the pair-fed birds in experiment 8. The similarity in the growth and EFC of chicks fed both diets in experiment 8 is a strong indication that mimosine and/or 3,4-DHP and their potential metabolites, restrict growth by interefering in the biochemistry of food intake. This interference may be associated with phenylalanine and tyrosine amino acid metabolism and their involvement in food intake regulation in the brain.

The subject of amino acid interactions and their effects on food intake and neurotransmitter levels is the subject of much study (Peters and Harper, 1985; Tews and Harper, 1985; 1986a; 1986b) and thorough review of the literature on this topic is currently in preparation (L. M. Harrison PhD Thesis, personal communication).

Dietary amino acid imbalances, and the effect of inclusion of amino acid analogues in rat diets has been shown to inhibit growth and food intake in rats (Harper, Becker and Stucki, 1966; Tews and Harper, 1985). The adverse effects of incorporation of abnormal amino acids and abnormal relative concentrations of amino acids into diets may be attributable to the alteration of the relative levels of amino acids in serum and in the brain (Harper, Becker and Stucki, 1966; Tews, Bradford and Harper, 1981a; 1981b; Tews and Harper, 1983; 1985; 1986a; 1986b). Similarly, rats fed a diet containing mimosine at 40g kg^{-1} had reduced growth rates relative to controls (Lin, Lin and Tung, 1964). It may be assumed therefore that mimosine and/or its metabolites, because of their structural similarity (Fig. 1.9.3.1; 4.4.3), may significantly alter the concentrations of phenylalanine and tyrosine and their metabolites, DOPA, dopamine and norepinephrine (Fig. 4.4.3; 4.4.4) in the brain thus reducing food intake and growth rate. The concentrations of the catecholamines are likely to be influenced by either interference in their synthesis (Fig. 4.4.3) or catabolic degradation (Fig. 4.4.4; sect. 1.9.3.2). These neurotransmitter compounds have been shown to influence food intake (Grossman, 1962; Kruk, 1973; Ritter and Epstein, 1975).

Addition of phenylalanine and tyrosine to rat diets containing LLM has been shown to ameliorate to some extent the adverse effects on growth caused by the presence of mimosine (Lin, Lin and Tung, 1964). The beneficial effects may be due to a competitive interaction of these amino acids with mimosine and/or its metabolites. This may reduce any interference of mimosine and/or its metabolites on brain catecholamine levels by reducing the amount of mimosine in the brain rather than increasing the amount of the catecholamine precursors, tyrosine and phenylalanine present which appears to have little effect on brain catecholamine

levels in mice and rats (Peters and Harper, 1985; Johnson, Romsos and Bergen, 1986; Tews and Harper, 1986a). This may indicate that mimosine and/or its metabolites may, indeed be extremely potent once in the brain because of its close structural similarity to DOPA. The lack of detectable quantities of mimosine in the blood (chapter 3) does not support this hypothesis and is contrary to that of other unconventional amino acids consumed by rats and also those levels of canavanine found in chick serum after the consumption of JB or canavanine (chapter 3; Tews and Harper, 1986a; 1986b).

The lack of effect of mimosine and/or its metabolites in the hepatic system is substantiated by the relatively small changes in serum ALT and AST activities which are within normal ranges for poultry (McDaniel and Dempsey, 1962; Grunder, Lefkovitch, Chambers and Gavora, 1983; Table 3.3.19). If mimosine was present in the serum in substantial quantities it might be expected that ALT and AST activities would decrease due to the potent effect of mimosine on such pyridoxal phosphate requiring enzymes (Serrano, Ilag and Mendoza, 1983; sect. 1.9.3.2). Perhaps mimosine and 3,4-DHP are transported to the brain in quantities lower than those detectable by UV detection or the small quantities which may be present are lost during sample preparation (Table 3.1.2). The use of ECD with HPLC will assist in elucidating these hypotheses.

As well as the induction of imbalances of amino acids in vivo by mimosine it may be that the inhibition of enzymes such as the aromatic amino acid decarboxylase (Lin, Lin and Ling, 1963; Borchardt, 1973; Hashiguchi and Takahashi, 1977; Grove, Ballata, Eastmo and Hwang, 1978; Fig. 4.4.3) is a contributary factor in affecting the levels of the neurotransmitters and thus feed intake. It is also possible that mimosine and/or its metabolites, is incorporated into enzymes in a similar manner to that reported for canavanine (Crine and Lemieux, 1982) thus reducing their effectiveness. This assumption is less probable than that for canavanine since mimosine is less like phenylalanine and tyrosine than canavanine is to arginine.

Ongoing work should shed light on the relationship of mimosine and its metabolites to the neurotransmitter levels and also to the interaction with normal physiological amino acids in the brain and serum.

4.4.6 Experiment 6.

The severe growth depression of chicks fed the LLM diets (6.2 and 6.3) which was accompanied by low EFC values, is likely to be due to reduced energy intake caused by the low dietary AME values (Table 3.3.20). Palatability problems

associated with the high inclusion level of LLM and thus tannins and saponins, are also likely to have exacerbated the problem. The mimosine intake related to bodyweight, of chicks was relatively low in comparison with those of other experiments described here. Comparison of growth rates, EFC and ENR in this experiment with those in the lupin experiment (experiment 13) where lupins were substituted at the same level as LLM in this experiment, shows that RGR values were much higher for the lupin fed chicks as was dietary AME and EFC. The ENR values for lupin fed birds in experiment 13, approached those for LLM fed birds in this experiment.

Although the WSC content of LLM increased with preincubation of LLM with hemicellulase, the enzyme treatment did not improve the AME values of LLM, either when LLM was fed alone or as a dietary component, and in fact they were depressed in comparison to the values which have been published (D'Mello and Thomas, 1978; D'Mello and Acamovic, 1982b). The complete lack of a beneficial effect here was in contrast to the positive responses gained with chicks fed diets containing guar gum and a hemicellulase fraction (Ray, Pubols and McGinnis, 1982).

It appears that further treatment of LLM is necessary in order to reduce the adverse effects of the carbohydrates and polyphenolics in LLM, on gut enzymes (Marquardt and Ward, 1979, Griffiths, 1979; Griffiths and Moseley, 1980; Deshpande, Sathe and Salunkhe, 1984) thereby increasing the AME value of LLM.

4.4.7 Experiment 7.

Dietary tannin supplied from sorghum, in the sorghum diets (7.2, 7.3 & 7.4), was higher than that supplied from LLM in all the LLM based diets but the RGR for chicks fed the sorghum basal diet was higher (Fig. 4.4.1) than for chicks fed any of the LLM diets. From the information in the literature (Hewitt and Ford, 1982; Banda and Vohra, 1983) and from the LLM experiments reported here the lack of growth depression caused by inclusion of sorghum, with a tannin content of 22.5g kg^{-1} DM, was surprising. The depression in growth and food intake caused by the addition of Fe(III)) to these sorghum diets (diets 7.3 & 7.4) was probably due to the reduction of pH (Table 2.13.9) associated with supplemental Fe(III). It would appear that the tannins in sorghum are less potent in their adverse effects than those in LLM. In other work, however, sorghum tannins have been reported to have similar chemical and physical characteristics to those in the faba bean yet still have greater potency than faba bean tannins

(Strumeyer and Malin, 1975; Marquardt, Ward, Campbell and Cansfield, 1977; Ford and Hewitt, 1979c).

4.4.8 Experiment 8.

Discussion of the results of this experiment has been presented in section 4.4.5. The reduced ENR for mimosine fed birds compared with the control birds is likely to be due to the high efficiency with which chicks excrete mimosine and its metabolites. Data on the effects of mimosine on catecholamine and brain and serum amino acid levels remain to be obtained as has the efficiency with which mimosine is excreted. The efficiency of excretion of mimosine from a cockrel fed mimosine has been reported to be negligible (Springhall, 1965) although that for LLM fed chicks is high (Tables 3.3.5, 3.3.10 & 3.3.12; D'Mello and Acamovic, 1982). It is notable that mimosine has no effect on the AME of the diet, either in experiment 5 or 8, which indicates that it does not interact adversely with energy utilising gut enzymes.

4.4.9 Experiment 9.

Feeding raw JB to animals can kill them (sect. 1.10.3). As described and discussed previously, cooking increases the nutritional value of legumes and reduces the effects of heat labile antinutrients.

Incorporation of JB, which were autoclaved primarily to destroy the activity of the Con A and other lectins (Carlini and Guimaraes, 1981) but also to reduce the trypsin inhibitor activity (Table 2.8.1) and the a-amylase inhibitor activity (Jaffe, 1973), at 140g kg^{-1} diet did not support growth to the same extent as the control diet (diet 9.1). Such a response is in agreement with earlier work where autoclaved JB was fed to chicks (D'Mello, Acamovic and Walker, 1985). Interestingly the RGR for the JB basal diet (9.2) was about the same as that obtained for chicks fed the LLM basal diets (Fig. 4.4.1; 4.4.4) The relative canavanine and mimosine intakes for chicks fed the respective diets were also about the same. The major nutritional difference between LLM and autoclaved JB and lupins, is the large discrepancy in their AME values (Tables 2.13.2, 2.13.7, 2.13.16, 2.13.27; D'Mello and Acamovic, 1982; D'Mello, Acamovic and Walker, 1985). The AME of LLM is much lower than JB and lupin despite the similarity in their fibre contents. The higher AME of JB compared to LLM is reflected in the lower oil additions to the JB diets than the LLM diets.

If dietary canavanine from the autoclaved JB is considered as the growth depressing factor in chicks fed JB it seems probable that canavanine, in a

similar way to homoarginine in rats (Tews and Harper, 1983; 1986a; 1986b), interferes with lysine and arginine balances in the chick.

The interrelationship between arginine and lysine in poultry is well documented (Lewis, Smith and Payne, 1963; Smith and Lewis, 1963; Boorman and Fisher, 1966; Jones, Petersburg and Burnett, 1967; D'Mello and Emmans, 1975; Visek, 1986). Arginine and lysine have also been shown to have a similar relationship in the rat where work has demonstrated that arginine and its analogues are effective in reducing lysine transport to the brain when included in diets at high concentration (Tews, Bradford and Harper, 1981a; Tews and Harper, 1983). Inclusion of homoarginine in rat diets for short term feeding experiments, causes a depression of serum lysine concentrations and also induces low brain arginine and lysine levels (Tews and Harper, 1986a). Longer term experiments demonstrated that rats had depressed growth rates and food intakes, while serum lysine and ornithine but not arginine, concentrations were depressed when homoarginine was included in the diet (Tews and Harper, 1986b).

When chicks are fed diets containing autoclaved JB, as in the experiments reported here, they may be unable to distinguish between canavanine and arginine and therefore the sum of canavanine and arginine may be biochemically and physiologically interpreted as total arginine thereby inducing an imbalance between arginine and lysine. Lysine would then appear to be low relative to arginine, in a similar manner to the effect of including homoarginine in rat diets (Tews and Harper, 1986b).

Dietary supplementation of a chick diet with lysine (diet 9.3) did not redress the growth rate or food intake to that of birds fed the control diet (Table 3.3.22; Fig. 4.4.4). Indeed additional lysine reduced the growth and food intake further (Table 3.3.22). This is contrary to the effect of supplementary lysine on rats fed diets containing homoarginine (Tews and Harper, 1986b).

The improvement in performance (Table 3.3.22) of birds fed a JB diet with supplemental arginine (diet 9.4) is also in direct contrast with rats fed homoarginine (Tews and Harper, 1986b) but in agreement with previous work with JB fed to chicks and on the beneficial effects of arginine on canavanine inhibited bacteria (Volcani and Snell, 1948; D'Mello, Acamovic and Walker, 1985). The implication from the response obtained in this experiment is that canavanine competes with arginine thereby causing an apparent imbalance in the favour of lysine. Thus supplemental arginine redresses the balance to some extent while additional lysine enhances the imbalance by increasing arginase activity, thus increasing the requirement for arginine (Lewis, Smith and Payne,

1963; Smith and Lewis, 1963; Sato, Nakaya and Itoh, 1982). Increased arginase activity in chicks fed the lysine supplemented JB diet (9.3) may be assumed from the slight increase in serum urea concentrations. It is notable that serum urea concentrations doubled (Table 3.3.23) when chicks were fed the JB basal diet rather than the control diet. This indicates that the chick appears to regard the canavanine and arginine present as total arginine thus causing increased production of urea in agreement with dietary increases in arginine in this experiment and elsewhere (Sato, Nakaya and Itoh, 1982). The reasonable stability in serum NH3 concentrations is in accord with that reported elsewhere and is in agreement with the very low levels of urease in the chick (Sato, Nakaya and Itoh, 1982).

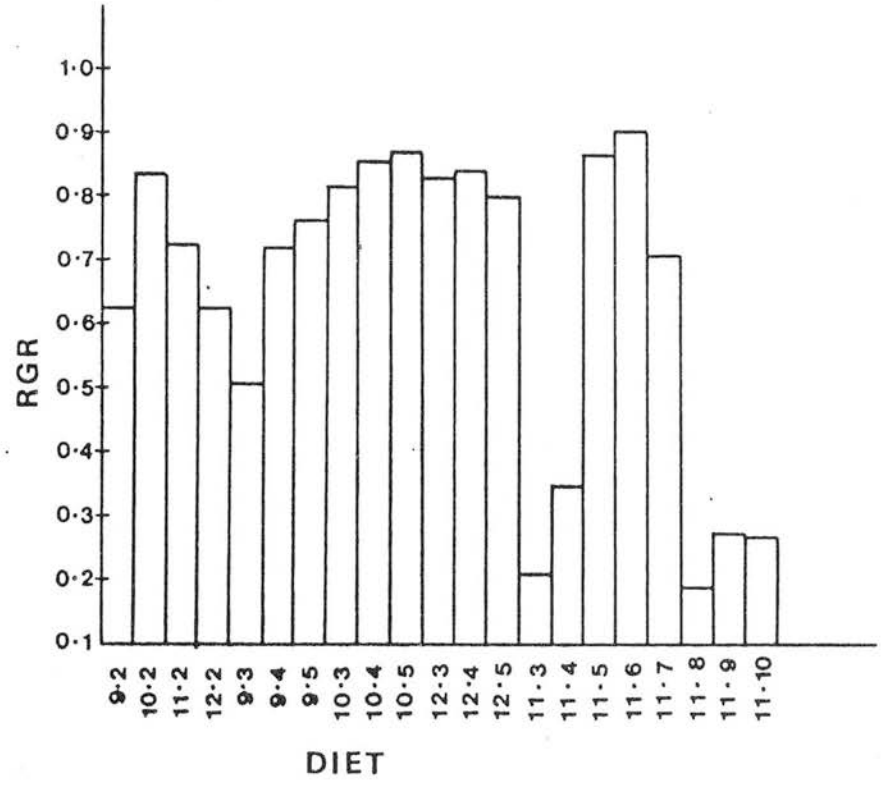
Competition between arginine and canavanine would inhibit the degradation of arginine by arginase in the liver and kidney by reducing the amount of arginine able to associate with the enzyme. On the other hand, arginase activity would be reduced in proximity with canavanine because of the slower degradative effect of the enzyme on canavanine than on arginine (Damodaran and Narayanan, 1940; Rosenthal, 1977; sect. 1.10.4). The interference and degradation of canavanine would then affect the concentration of degradative products of arginine within the animal (Fig. 1.10.4). It might be expected that ornithine and other urea cycle intermediates would decrease due to the reduced degradation of arginine and also as a result of production of their analogues.

This theory agrees with the results of feeding homoarginine to rats. In that experiment ornithine levels in serum dropped when homoarginine was included in the diet (Tews and Harper, 1986b). Since urea is a common degradation product of both arginine and canavanine it is reasonable that there is an increase in urea production when JB is incorporated into the diet (Table 3.3.23).

It is also possible that the ingested canavanine which, in apparent contrast to mimosine, enters the peripheral blood (Table 3.3.23) in which it is transported to the brain and other organs where it is incorporated into neural or other proteins. These proteins may then have reduced functional capability in accord with other work (Noe, 1981; Crine and Lemieux, 1982, sect. 1.10.4).

Small quantities of canaline, which have not yet been detected in serum (sect 4.4.1), may also be transported to the brain and there, may interfere with neurotransmitter levels by inhibition of enzymes such as 5-hydroxytryptophan decarboxylase (Rahiala, Kekomaki, Janne, Raiana and Raiha, 1971). This would then cause similar effects as those proposed for mimosine and its metabolites (sect. 4.4.7).

Fig. 4.4.5 Relative growth rates (RGR) of chicks fed jack beans



RGR = Growth Rate / Growth rate of chicks fed the control diet for each experiment

The small response in growth and EFC on addition of both lysine and arginine may be due to the production of a better balance between these amino acids in the diet.

Further work is required to ascertain the presence or absence of canavanine, canaline and their metabolites in the various tissues of the chick. The use of HPLC to identify and quantify these analogues and their conventional arginine analogues and thus obtain their relative concentrations will assist in elucidating the mechanism of toxicity of canavanine within the chick.

4.4.10 Experiment 10.

The slightly better growth rates (Fig. 4.4.5) and food intakes of birds fed JB diets in this experiment may be partly due to the different pretreatment of the JB which resulted in a small decrease in canavanine content of the treated JB (Table 2.13.18) and which may have increased the nutritional value of the protein and carbohydrate as discussed earlier.

It is remarkable, considering the foregoing discussion and the experimental evidence with rats (Tews and Harper, 1986b), that supplemental ornithine had no beneficial effect on chick growth. It may be that the supplemental level of ornithine was inadequate or excessive. As in experiment 9, supplemental arginine, and arginine and lysine had slight beneficial effects (Table 3.3.24) but none of the amino acid combinations improved growth to those of control fed birds. The implication is that the other antinutritional factors such as tannins and saponins, and perhaps carbohydrates, exert their effects on growth. This may not, however, be an accurate assessment of the picture since growth rate of rats fed diets containing homoarginine did not grow to their full potential on supplementation with lysine (Tews and Harper, 1986b). The mechanism involved seems, however, to be different in both cases.

The poorer ENR values in this experiment compared with those in experiment 9 are no doubt due to the higher quantity of glutamic acid included in the diets of this experiment.

4.4.11 Experiment 11.

Germination of JB has been reported to reduce canavanine levels (sect. 1.10.4; Ho and Shen, 1966; Rosenthal, 1977). It was therefore surprising to find that of all the treatments of JB, the germinated beans, after soaking and autoclaving, had

the highest canavanine contents (Table 2.13.21). It was also expected that the other nutritional benefits of germination and cooking would be reflected by superior growth responses. The benefits of cooking have been discussed in other sections of the work reported here.

Germination of legumes has been shown to be successful in reducing the content of oligosaccharides, trypsin inhibitors, lectins and tannins in legumes as well as to increase the free amino acid and mono and disaccharide contents (Palmer, McIntosh and Pustzai, 1973; Ologhobo and Fetuga, 1984 Crawshaw and Reid, 1984; Jood, Mehta, Singh and Bhat, 1985; Bednarski, Tomasik and Piatkowska, 1985). Similar occurrences have also been shown for sorghum (Elmalik, Klopfenstein, Hoseney and Bates, 1986). Although in some cases, nutritional quality has been improved by germination, feeding uncooked material has not always produced an increase in growth (Palmer, McIntosh and Pusztai, 1973; Elmalik, Klopfenstein, Hoseney and Bates, 1986) and sometimes adverse effects on nutritional quality and growth responses are observed (Elias, Conde, Munz and Bressani, 1975).

The dual advantages of germination and autoclaving were expected to produce good performances in chicks fed diets with JB treated in this manner. The extremely poor growth rates and general response (Table 3.3.25) was similar to that which had been reported previously, with JB fed at the same level (D'Mello, Acamovic and Walker, 1985). Germination followed by autoclaving did not enhance the nutritional value of JB.

The very much reduced canavanine content of the extracted JB (Table 2.13.21) did not allow chicks fed the diet containing this material (diet 12.5) to perform at the same level as control fed birds. This is indicative of the presence of other antinutritional factors such as tannins and saponins. This assertion is supported by the better than expected performance of birds fed the canavanine supplemented diet (diet 10.6). Although birds fed the canavanine supplemented diet had a poorer performance than the control fed birds the response did not correspond with the canavanine intakes or levels in the diets. This appears to be in agreement with the inability of arginine to alleviate, completely, the deleterious effects of JB in the diets (Tables 3.3.24 & 3.3.28).

Although heating at 100°C reduced the apparent canavanine content, presum ably due to Maillard reactions, of JB by about 110g for each kg of canavanine present in JB (Table 2.13.21), performance of chicks fed diets containing this material (diets 10.7 & 10.8) was not improved, perhaps due to the adverse effects of treatment at high temperature. The slight improvement

associated with soaking whole beans is probably due to losses of antinutritional factors during soaking after which the steeping water was discarded. The discarded water is discoloured and soapy in nature which is consistent with the presence of saponin and proteinaceous material. Longer soaking at higher temperatures has been shown to enhance this effect (D'Mello, personal communication). Therefore, the small discrepancy between chicks fed the ground and soaked beans and those fed the beans which were soaked whole is likely to be due to losses in the discarded washings. Although there is a large reduction in canavanine contents between beans dried at 60 and 100°C (Table 2.13.21) animal performance does not reflect this. The adverse effects of heating at 100°C may have detrimentally influenced the response of chicks fed diets containing JB dried at 100°C.

Chicks in this experiment had similar serum canavanine concentrations, except those fed the diet containing added canavanine (diet 9.6), to those in experiment 9. In both these experiments and in experiment 12, canavanine levels in serum are much lower than those for serum homoarginine in rats fed diets containing 19.2g kg^{-1} of homoarginine (Tews and Harper, 1986b). The serum level of canavanine reported here for chicks fed canavanine added to the diet at 4g kg^{-1} diet (Table 3.3.26) was about one third of that for homoarginine in rats (Tews and Harper, 1986b). The lower canavanine levels found in the serum of chicks fed JB or canavanine in the work reported, compared to the levels of homoarginine and other unnatural amino acids found in rat blood, is no doubt due to the difference in intakes (Tews and Harper, 1986a; 1986b).

Dietary canavanine levels, canavanine intake or serum levels of canavanine did not have an effect on serum NH_3 levels in experiments 9, 11 and 12. This is consistent with the lack of urease present in the chick as discussed earlier (sect. 4.4.9). It may have been expected that urea in the serum of chicks fed the canavanine supplemented diet (diet 11.6) would have been higher due to the higher canavanine content in the serum. The lack of a substantial increase may be due to the higher concentration of canavanine causing substantial deactivation of arginase as discussed earlier.

The canavanine content of serum did not correlate with the normalised intakes (Table 3.3.27) particularly for chicks fed the diet with added canavanine (11.6). This is in contrast with the serum canavanine levels obtained in experiments 9 and 12 in which the normalised canavanine intake was essentially directly related to serum concentrations (Tables 3.3.23; 3.3.27 & 3.3.30). The differences in this experiment may be due to the variation of treatment and therefore variation in

the presence of the other antinutritional factors in JB as well as the availability of canavanine.

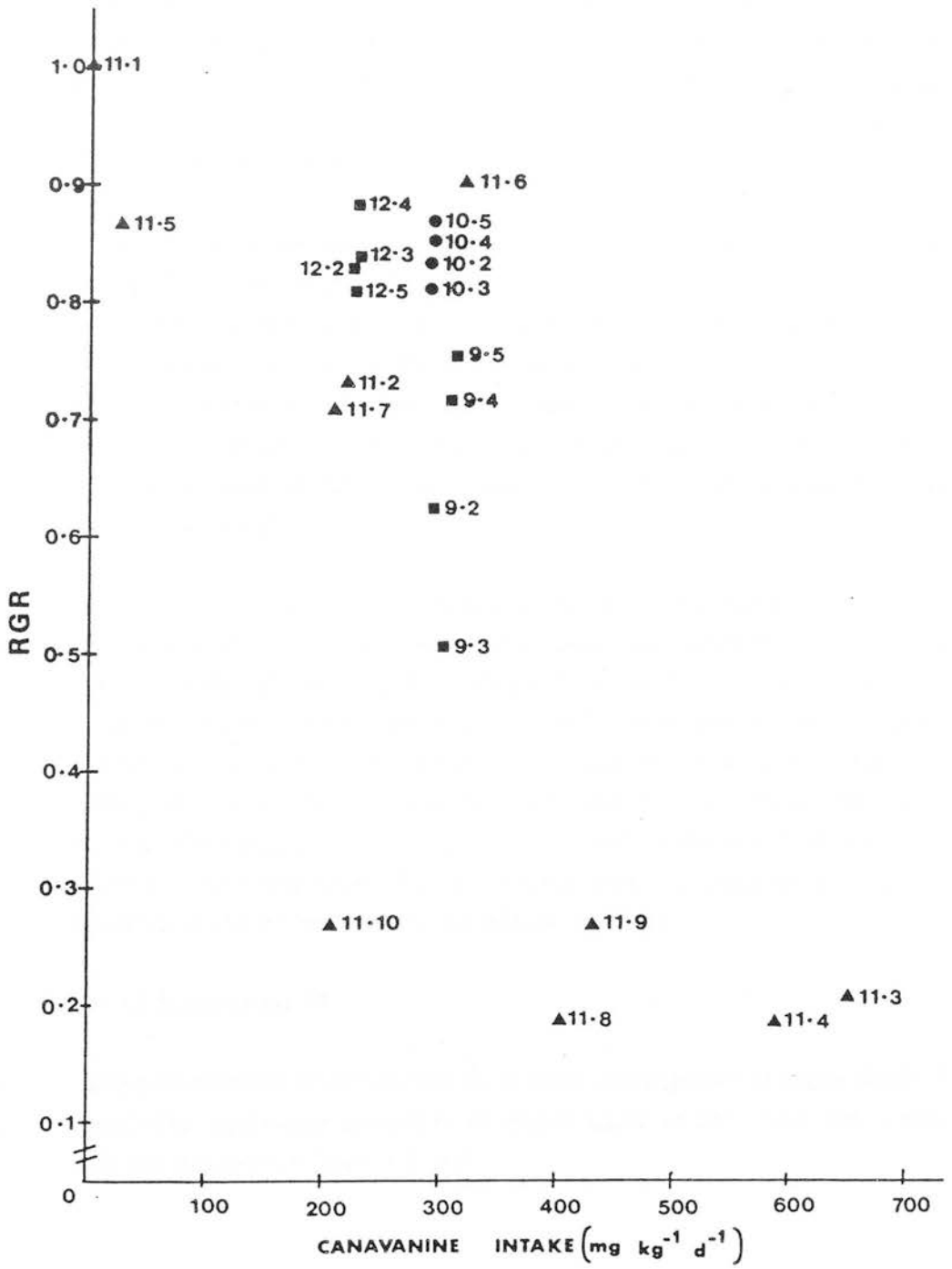
The lack of variation in AST and ALT activity in the serum is indicative of the lack of effect of JB and canavanine, or its metabolites, on these enzymes and is in contrast to the effect of feeding raw lima beans, on AST and ALT activity in rats (Aletor and Fetuga, 1984). The lack of effect of diet on ALT and AST activity in chicks in the experiments reported here, also tends to confirm the finding with HPLC that canaline, which is a potent inhibitor of such enzymes, is absent, or present in very small quantities in the serum. The activities of ALT and AST are similar to those reported for mimosine fed chicks of a similar age and those chicks in experiment 12 (Tables 3.3.19 & 3.3.29). The activity of ALT and AST is also of the same order as those reported elsewhere for poultry (McDaniel and Dempsey, 1962; Grunder, Lefkovitch, Chambers and Gavora, 1983; Mohammed, Dessouky, Hussein, Mohhamed and Sokar, 1983) and is indicative of lack of liver damage or enzyme inhibition.

4.12 Experiment 12.

In this experiment the supplementation of equinitrogenous amounts of arginine and lysine to JB diets (Table 2.13.22) gave better responses than the chicks in experiment 9 (Tables 3.3.22 & 3.3.28; Fig 4.4.5). Interestingly the RGR values of birds fed the JB basal diets (diets 9.2 & 12.2) were the same although dietary canavanine levels were different and normalised canavanine intakes were also different from each other. The JB pretreatment is considered to be the cause. In experiment 9 the whole JB was, effectively, washed whereas in this experiment the ground beans were soaked but not washed which would tend to allow other heat stable antinutrients to persist in the beans.

The response of birds to the diets with the low level of addition of arginine and lysine (diet 12.3) was as good as their response to the other levels of addition (Table 3.3.28) which may indicate that the remaining growth depression is due to other antinutritive factors. This tends to agree with the results obtained for experiment 11 where canavanine supplementation did not produce such severe effects as the JB basal diet and where the diet containing the residual JB after extraction, did not allow birds to perform as well as control fed birds. As in experiment 9 the increased urea concentrations in the serum is in agreement with increased arginine intake (Sato, Nakaya and Itoh, 1982). The lower intakes of canavanine in this experiment compared with those in experiment 9 is the likely cause of the reduced serum canavanine levels observed (Table 3.3.29).

Fig. 4.4.6 Plot of Canavanine intake (mg kg^{-1} liveweight d^{-1}) vs. relative growth rate (RGR) of chicks fed diets containing jack beans.



It is remarkable that there is relatively little variation in serum canavanine contents between all the chicks receiving the diets containing JB and that very little variation is caused by additional lysine or arginine or by canavanine intake (Tables 3.3.23; 3.3.26; 3.3.29). This observation is in agreement with that for rats fed diets containing homoarginine (Tews and Harper, 1986a; 1986b).

From the work presented here (Fig. 4.4.6) it is apparent that consumption of JB, such that normalised canavanine intake is greater than about 25mg kg^{-1} liveweight d^{-1}, causes reduced RGR in chicks. This is more severe than the consumption of mimosine (Fig. 4.4.2). The addition of arginine to diets allows a greater intake of canavanine before the growth is depressed to the same extent as the unsupplemented diets (Fig. 4.4.6). From the results obtained here it cannot be concluded that canavanine is the only toxic or antinutrient present in autoclaved JB. Indeed it is apparent that there are other compounds present which adversely affect growth of chicks.

The intriguing interrelationship of canavanine and its metabolites with serum and brain amino acids, particularly those involved in the arginine-urea cycle (Fig. 1.10.4), await to be unravelled when the HPLC methodology is sufficiently refined so as to permit the identification and quantitation of these compounds. In a similar manner to mimosine, the effects of the consumption of canavanine on brain and catecholamine levels may reveal the nature of the toxicity of canavanine. Analysis of catecholamines using HPLC will also assist in elucidating the mechanism of toxicity.

The animal experiments necessary to ascertain the mechanism of toxicity of canavanine, and mimosine, require that pure compounds be fed at various levels and the fate of the ingested amino acids and their metabolites monitored carefully using HPLC techniques. Such experiments would provide an opportunity to examine the effects of the free amino acids on their conventional analogues and on the synthesis and degradation of catecholamines in the brain without the confounding effects of other toxic compounds in the parent plant material. Large quantities of plant material would be required to isolate sufficient amounts of the amino acids to include in the diets.

4.4.13 Experiment 13.

The performance of chicks fed lupin diets was superior to those chicks fed diets containing equivalent quantities of either LLM or JB. From the literature this was not unexpected (sect. 1.11.3).

The poorer performance, relative to control fed birds (Table 3.3.13) caused by inclusion of uncooked lupin at 400g kg^{-1} is in agreement with other workers (King, 1981; Halvorson, Shehata and Waibel, 1983; Karunajeewa and Bartlett, 1985). The poor performance is likely to be associated with the presence of small amounts of alkaloids as well as the antinutritional effects of the carbohydrates present in lupins (Brillouet, 1984; Carre and Leclercq, 1985; Carre and Brillouet, 1986). The presence of saponins and low levels of erucic acid and canavanine are also likely to exacerbate the deleterious effects of lupin on chick performance (Hudson, 1979; sect. 4.2 & 4.3).

The improvement in performance obtained by autoclaving the lupin and including it at 200g kg^{-1} diet is indicative of the destruction of heat labile antinutrients as well as increasing the available nutrient content and reducing the presence of antinutritional carbohydrates (Reddy, Pierson, Sathe and Salunkhe, 1984; Mehta, Singh and Bhat, 1985; sect. 4.4.5). This is contrary to the conclusion arrived at in other work where pigs were fed heat treated lupins (Batterham, Andersen, Burnham and Taylor, 1986).

The improvement in performance obtained for the low level of addition of autoclaved lupin was not attained when this material was included at 400g kg^{-1}. Indeed performance for birds fed this material was slightly lower than for birds fed the same level of uncooked lupins (Table 3.3.31). The lack of a positive response at the high inclusion rate is, no doubt, due to the adverse effects caused by browning of the material during drying at 60°C. The adverse results obtained for pigs which were fed dry heat treated lupins and autoclaved lupins, without prewetting, which were oven dried for 2h prior to grinding and inclusion in the diets were more severe than that reported here for chicks (Batterham, Andersen, Burnham and Taylor, 1986; Batterham, Andersen, Lowe and Darnell, 1986). In those experiments high temperature dry heating influenced the availability of amino acids but autoclaving did not. Period of heating also influenced growth and EFC of pigs.

The poorer EFC and ENR values obtained for chicks fed diets containing autoclaved lupin at both levels, compared with birds fed the equivalent diets with unheated lupin, is undoubtedly due to the heat treatment and probably to the occurrence of Maillard reactions and the products of such reactions. Although browning reactions were obvious the similarity in growth of the chicks fed the heat treated or untreated lupins is evidence that amino acid availability was not a major problem.

In this experiment autoclaving of the ground seed caused a slight reduction in the AME(c) value compared to the value obtained for uncooked lupin which was in close agreement with previously published AME(c) values (Guallaume, Chenieux and Rideau, 1979; Halvorson, Shehata and Waibel, 1983). This is contrary to the findings of other workers who found that autoclaving lupins and other legume seeds, improved the true metabolisable energy (TME) and AME value of these for poultry (Shannon and Clandinnin, 1977; Molina, Sanz, Boza and Aguillera, 1983; Nwokolo and Oji, 1985; Boldaji, Goeger, Nakaue, Savage and Arscott, 1986). The imrovement in AME found by other workers on autoclaving, is likely to be due to the beneficial effects on the lupin carbohydrates perhaps making them more available and also by reducing the 'sweep out' effect by them (Walter, 1985). The reduction in AME in this experiment due to autoclaving, is probably attributable to the reduction in availability of carbohydrate caused by Maillard reactions which occurred during drying although the same effect did not appear in other legumes treated similarly (Nwokolo and Oji, 1985). Browning reactions were not reported in that experiment.

4.4.14 Experiment 14.

Treatment of the lupins with the enzyme mixture increased the content of low molecular weight carbohydrates (Fig. 3.3.1) indicating that higher molecular weight carbohydrates (Brillouet, 1984, Carre, Brillouet and Thibault, 1985) were degraded. This increase in the concentration of lower molecular weight carbohydrate was not reflected in better animal performance (Table 3.3.33). The poorer performance associated with the inclusion of preincubated lupin in chick diets is probably due to the formation of Maillard products during the drying procedure. The browning effect on lupins was more pronounced in this experiment than in experiment 13 and is likely to be due to the increased amount of low molecular weight carbohydrate and its greater exposure to the atmosphere caused by drying the ground sample.

The reduced performance of chicks in this experiment, compared with those in experiment 13, on heating may be associated with a reduction in the availability of amino acids as well as the formation of antinutritional products caused by browning (sect. 1.8). The reduction in ENR and EFC is in agreement with that reported for pigs (Batterham, Andersen, Burnham and Taylor, 1986; Batterham, Andersen, Lowe and Darnell, 1986) and may support this proposition.

Lyophilisation or perhaps spray drying would solve the problem of formation of undesir able compounds during the drying process and would thus enable

unconfounded results to be obtained. The small increase in AME associated with preincubation of the lupin with the lower level of enzyme (diet 14.4) may be indicative of an increase in AME of the lupin due to enzymatic degradation of poly- and oligosaccharides but which is considerably masked because of the drying procedure.

Better post incubation treatment and variation of the components of the enzyme mixture and their concentration as well as variation in incubation time appears to have potential in increasing the AME value of lupins for poultry and perhaps pigs where drying may not be necessary.

4.5 Conclusions and future work

4.5.1 Conclusions and Summary of current work

The development of HPLC techniques for the analysis of canavanine, canaline, mimosine and 3,4-DHP during the course of the work presented here permitted the analysis of these compounds in chick excreta and serum as well as in the respective plant materials containing these. Neither free canavanine nor canaline was detected in the excreta of chicks fed JB or canavanine but almost all the mimosine and 3,4-DHP ingested by chicks fed LLM diets was excreted both in the absence and presence of supplementary dietary iron. Conversely, the serum from chicks fed diets containing LLM did not show the presence of mimosine or 3,4-DHP while the serum of chicks fed diets containing JB or canavanine contained detectable quantities of canavanine but not canaline. The precipitation of protein in serum caused losses of added mimosine and 3,4-DHP but not canavanine or canaline. Using the HPLC technique it was ascertained that canavanine levels in JB did not vary extensively during germination.

Saponins were detected in extracts of LLM, LS, JB, lupin seeds and soya beans when the ethanol extracts of these materials were analysed using TLC. Tannins were also determined to be present in these legumes although soya beans were not tested for the presence of tannins. Lupin seeds did not contain detectable quantities of trypsin inhibitors while the other legumes did.

Enzyme treatment of both lupins and LLM increased the quantity of lower molecular weight carbohydrates present in these materials but had little effect on the AME of lupins and no advantageous effects with LLM.

Inclusion of canavanine or mimosine in chick diets at similar levels to those in diets which contained JB and LLM respectively did not induce such severe depressions in performance. The obvious implication from this finding is that

mimosine and canavanine are not the sole antinutrients present in LLM and JB respectively.

Incorporation of LLM into poultry diets caused substantial depressions in growth. Addition of Fe(III) produced the largest response in the performance of chicks fed diets containing LLM compared to untreated LLM containing diets. Other treatment of LLM such as heating the LLM prior to incorporation into the diet also had a substantial beneficial effect on growth while addition of PEG4000 and cholesterol enhanced the responses to Fe(III) and heat treatment of LLM.

Addition of lysine and ornithine to JB diets had a negligible effect on chick performance while addition of arginine improved performance. Additional dietary arginine also reduced the amount of detectable canavanine present in the serum of chicks fed JB diets supplemented with arginine.

4.5.1 Future work

Effort is required in order to further develop the HPLC methods of analysis of mimosine and 3,4-DHP and the metabolites of these, in the physiological fluids of poultry which has consumed these compounds. Further development of the HPLC method with EC detection will enhance our understanding of the metabolism of these compounds within poultry and probably also within sheep, cattle and other ruminants. The enhanced sensitivity of EC detection may assist in monitoring low levels of such compounds within the animal and in the excreted material as well as assisting in determining whether mimosine and 3,4-DHP are present either free or in conjugated form. HPLC techniques are likely to help elucidate the effect of ingestion of mimosine and 3,4-DHP on catecholamine synthesis and catabolism within animals consuming mimosine, 3,4-DHP and *Leucaena*. Monitoring the effects of mimosine and its metabolites on the catecholamines may shed light on whether mimosine exerts some of its effects by interference in food regulation. Conversely addition of mimosine and/or 3,4-DHP to diets may assist in determining the effects of catecholamines on food regulation. It may also be possible to ascertain if mimosine and/or its metabolites are incorporated into proteins in a similar manner to canavanine and if so, what effect this has on the functional capability of the proteins containing mimosine and its metabolites.

An interesting but difficult task where there is much scope for investigation is the characterisation and quantification of the saponins which appear to be present in LLM and LS. Combined with this, a study of the effects of these compounds on the nutritional quality of LLM and their effects *in-vivo* is

warranted as is the characterisation and nutritional evaluation of the tannins, carbohydrates and the lipids and their effects *in-vivo*.

The major limitation on using LLM, which has been supplemented with Fe(III), for use in poultry diets is the extremely low AME value. This is likely to be due to the presence of undigestible carbohydrates and perhaps lipids as well as the presence of saponins and tannins which are likely to cause a sweep out of bile acids and other lipids. Studies require to be conducted in order to ascertain the reasons for the extremely low AME of LLM and are likely to include enzyme supplementation of the diets or the LLM prior to incorporation into the diets.

Further studies on the nutritional consequences of the consumption of canavanine, without the confounding factors associated with jack beans, are necessary. This would entail the extraction and purification of large quantities of canavanine from jack beans and incorporating the canavanine at various levels in the diet with subsequent study of various physiological parameters. Canavanine, canaline and their conjugates would require to be monitored in the serum, excreta and in tissues such as brain and other tissue proteins, using the HPLC technique developed during the course of the work described here. The effect of the presence of such compounds on enzyme activity and on the amino acid composition, both free in the serum and in the tissue, and the effect on growth require to be studied. The presence or absence of canavanine in other members of the Papilionoideae such as lupins requires to be ascertained using HPLC because of its enhanced sensitivity compared to the PCAF technique which has been the conventional methodology used for this purpose.

In order to alleviate the problems associated with feeding JB to poultry and pigs, effort is required to effectively remove the antinutritional components of JB irrespective of their nature. This may be achieved by leaching the antinutrients from the beans using organic solvents, water or aqueous salt solutions. Germination of the beans requires to be studied further in order to assess the effects of germination under varying conditions of temperature and light, on the antinutritional factors present. Further work requires to be conducted on the effect of supplementing JB diets with various levels of arginine and, perhaps, lysine. Scope also exists for assessing the nutritional effect of adding other urea cycle products such as urea, to JB diets for chicks.

It is also imperative that the methods available for destruction of heat labile components be investigated thoroughly. The effectiveness of conventional boiling and drying for various periods of time and at various temperatures require to be investigated thoroughly in order to optimise the destruction of the various components but minimise the adverse effects of heating. Other techniques such as micronisation, microwave treatment and spray drying are also

worthy of investigation although such techniques may have limited availability in poorly developed areas of the world where solar methods of heating and drying may be studied.

The production of lupins within the UK and Europe for inclusion in animal diets has considerable potential particularly since there are relatively few antinutritional properties associated with these compared to the various other legumes which are currently produced. The financial support given by the EEC is also likely to encourage the production and use of lupins in Europe. Since the carbohydrates in lupins are not of equivalent nutritional quality to those from maize and thus are likely to contribute considerably to the poorer AME of lupins compared to maize, further work is necessary in order to improve the nutritional quality of the carbohydrates. This may be accomplished by conducting thorough studies on the effects of incubating the lupin seed meal with various enzymes and ascertianing which are the best enzymes and conditions necessary to degrade the oligomeric and polymeric carbohydrates present in lupins. It may, of course, be possible to include the enzymes dry in the diets containing lupins which would obviate the necessity to dry the incubated seed. Drying conditions also require to be studied in detail in order to minimise the formation of Malliard products. Freeze drying or spray drying may be beneficial in this respect. It may be that wet lupin seed which has been treated with the appropriate enzymes is a suitable dietary ingredient for pigs. In any case it would be essential to monitor the carbohydrate degradation products by HPLC or GC and to monitor their digestibility using the same techniques.

It must be remembered that fungal infection of lupins grown in the UK may occur and therefore the occurrence of lupinosis may be a problem. Work is required to ascertain whether any fungal contamination can be tolerated and if so what are permissable levels of the phomopsin toxins. Production of such toxins may limit the cultivation of lupins in the UK.

It may be of interest, in common with *Leucaena* and jack beans, to characterise the saponins present in lupins which may contribute to the relatively poor AME value of lupin seed for poultry. Further work is necessary in order to improve the AME of lupins for poultry. The concentrations and variety of alkaloids in lupins grown in the UK also require to be monitored and maintained at low levels. Another area of work which may be pursued with lupins is the bioavailability of the pigmenting material in the lupins for poultry, both for producing yellow pigmented carcasses and to enhance the yellow colour in egg yolks.

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SUMMARY OF DIETS FOR EXPERIMENTS 1 TO 8

Leucaena leaf and seed and sorghum

Diet

1.1 LLM 150
1.2 LLM 150 + PEG4000 (20g kg^{-1}).
1.3 LLM 150 + PEG4000 (40g kg^{-1}).
1.4 $Fe_2(SO_4)_3$ (24g kg^{-1}).
1.6 150 + PEG4000 (20g kg^{-1}) + $Fe_2(SO_4)_3$ (12g kg^{-1}).
1.7 LLM 150 + PEG4000 (40g kg^{-1}) + $Fe_2(SO_4)_3$ (24g kg^{-1}).
2.1 soya/maize control
2.2 soya/maize control + $Fe_2(SO_4)_3$ (4.8g kg^{-1}).
2.3 soya/maize control + $Fe_2(SO_4)_3$ (12.0g kg^{-1}).
2.4 LLM 150
2.5 LLM 150 + $Fe_2(SO_4)_3$ (1.6g kg^{-1}).
2.6 LLM 150 + PEG4000 (20g kg^{-1}) + $Fe_2(SO_4)_3$ (1.6g kg^{-1}).
2.7 LLM 150 + $Fe_2(SO_4)_3$ (4.8g kg^{-1}).
2.8 LLM 150 + PEG4000 (20g kg^{-1}) + $Fe_2(SO_4)_3$ (4.8g kg^{-1}).
3.1 soya/maize control
3.2 LLM 150
3.3 LS 65
3.4 LS 65 + $Fe_2(SO_4)_3$ (1.6g kg^{-1}).
3.5 LS 95
4.1 soya/maize control
4.2 LLM 150
4.3 LLM 150 + $FeSO_4$ (1.55g kg^{-1}).
4.4 LLM 150 + $FeSO_4$ (11.9g kg^{-1}).
5.1 soya/maize control
5.2 soya/maize control + PVP40 (10g kg^{-1}).
5.3 LLM 150
5.4 LLM 150 + PEG4000 (20g kg^{-1}) + $Fe_2(SO_4)_3$ (8.5g kg^{-1}) + cholest (5g kg^{-1}).
5.5 LLM 150 + PVP40 (10g kg^{-1}) + $Fe_2(SO_4)_3$ (8.5g kg^{-1}).
5.6 LLM 150 autoclaved
5.7 LLM 150 autoclaved + PEG4000 (20g kg^{-1}) + $Fe_2(SO_4)_3$ (8.5g kg^{-1}) + cholest (5g kg^{-1}).
5.8 soya/maize control + mimosine (3.3g kg^{-1}).
6.1 soya/maize/glucose control + $Fe_2(SO_4)_3$ (8g kg^{-1}).
6.2 LLM 400 + hemicellulase (3.3g kg^{-1}) + $Fe_2(SO_4)_3$ (8g kg^{-1}).
6.3 LLM 400 + hemicellulase (11.9g kg^{-1}) + $Fe_2(SO_4)_3$ (8g kg^{-1}).

7.1 soya/maize control

7.2 sorghum 300

7.3 sorghum 300 + $Fe_2(SO_4)_3$ (8g kg^{-1}).

7.4 sorghum 300 + $Fe_2(SO_4)_3$ (16g kg^{-1}).

8.1 soya/maize control

8.2 soya/maize control + mimosine (3.4g kg^{-1}).

SUMMARY OF DIETS FOR EXPERIMENTS 9 TO 12

jack bean

Diet

9.1 soya/maize control.

9.2 JB 140.

9.3 JB 140 + lysine (6.3g kg^{-1}).

9.4 JB 140 + arginine (6.0g kg^{-1}).

9.5 JB 140 + lysine (6.3g kg^{-1}) + arginine (6.0g kg^{-1}).

10.1 soya/maize control.

10.2 JB 140.

10.3 JB 140 + ornithine (1.5g kg^{-1}).

10.4 JB 140 + ornithine (1.5g kg^{-1}) + arginine (6.0g kg^{-1}).

10.5 JB 140 + ornithine (1.5g kg^{-1}) + arginine (6.0g kg^{-1}) + lysine (1.3g kg^{-1}).

11.1 soya/maize control.

11.2 JB 140, ground prior to autoclaving, dried at 60 C.

11.3 JB 280, germinated 3d.

11.4 JB 280, germinated 5d.

11.5 JB 140, extracted residue.

11.6 soya/maize control + canavanine (3.9g kg^{-1}).

11.7 JB 140, ground prior to autoclaving, dried at 100 C.

11.8 JB 280, ground prior to autoclaving, dried at 100 C.

11.9 JB 280, soaked, minced and aotoclaved, dried at 60 C.

11.10 JB 280, soaked, minced and autoclaved, dried at 100 C.

12.1 soya/maize control.

12.2 JB 140.

12.3 JB 140 + lysine (3.8g kg^{-1}) + arginine (3.6g kg^{-1}).

12.4 JB 140 + lysine (7.5g kg^{-1}) + arginine (7.3g kg^{-1}).

12.5 JB 140 + lysine (11.3g kg^{-1}) + arginine (10.9g kg^{-1}).

SUMMARY OF DIETS FOR EXPERIMENTS 13 and 14

lupins

Diet

13.1 soya/maize control.
13.2 lupin 200.
13.3 lupin 400.
13.4 lupin 200 autoclaved.
13.5 lupin 400 autoclaved.
14.1 soya/maize control.
14.2 lupin 400.
14.3 lupin 400 + dry enzymes (1.5g kg^{-1}).
14.4 lupin 400 pretreated with enzymes (1.5g kg^{-1}).
14.5 lupin 400 pretreated with enzymes (3.0g kg^{-1}).

Journal of Chromatography, 236 (1982) 169–179
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,385

DETERMINATION OF MIMOSINE AND 3-HYDROXY-4(1*H*)-PYRIDONE IN *LEUCAENA*, AVIAN EXCRETA AND SERUM USING REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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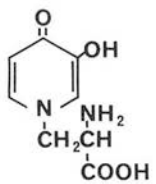
SUMMARY

The estimation of mimosine and 3-hydroxy-4(1*H*)-pyridone in *Leucaena leucocephala*, *Leucaena* seeds, chick excreta and chick serum using reversed-phase ion-pair high-performance liquid chromatography was investigated. Isocratic elution of both compounds was achieved in 11 min using sodium octyl sulphate as the pairing agent in a pH 2.25 buffer. Good recoveries of both mimosine and 3-hydroxy-4(1*H*)-pyridone in all but serum samples were obtained.

INTRODUCTION

The legume *Leucaena leucocephala* is grown extensively in semi-arid tropical and sub-tropical areas of the world, and its wide variety of uses have been thoroughly discussed[1–4]. Its use as a protein source (224–344 g kg^{-1} on a dry matter basis)[5,6] for animals is limited because of a number of factors, one of the major constraints being its relatively high content (10–100 g kg^{-1} of dry matter)[6,7] of the unusual and toxic amino acid mimosine ((*S*)-β-[N-(3-hydroxy-4-pyridone)]-α-aminopropanoic acid; I). It is well documented that mimosine is a depilatory agent, and studies on the use of mimosine and its analogues as defleecing agents have been reported in detail[8–10]. The large variety of biochemical, biological and nutritional effects of mimosine, including inhibition of protein synthesis, is also well documented[10–15,22,23].



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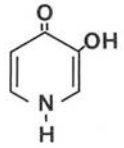

II

The major hydrolysis product of mimosine, 3-hydroxy-4(1*H*)-pyridone (DHP; II), has been found in dried *Leucaena*. It has also been found in ruminant and non-

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ruminant excreta when these animals have been fed diets containing *Leucaena* or mimosine[9,16–18]. DHP has been reported to be goitrogenic, a potent inhibitor of some enzymes[19,20] and a weak inhibitor of thymidine incorporation into mouse bone marrow cells *in vitro*[21].

The methods commonly used for the analysis of mimosine and/or DHP in *Leucaena* include colorimetry[24,25], paper chromatography[16], thin-layer chromatography[26], gas–liquid chromatography[27], ion-exchange chromatography (IEC)[28] and electrophoresis[29]. The use of IEC for the estimation of mimosine in ovine blood has also been reported[9,30]. All of these methods suffer from a variety of disadvantages, not least their inability to estimate mimosine and DHP simultaneously, rapidly, and in the case of colorimetry, specifically.

The use of high-performance liquid chromatography (HPLC) for the separation and estimation of amino acids from various sources is becoming popular[31]. Recently, however, the use of reversed-phase ion-pair HPLC (RP-IP-HPLC) for the separation and quantification of cation (and anion) forming compounds[32–34] has tended to predominate. A logical step in the estimation of both mimosine and DHP would, therefore, appear to be the use of RP-IP-HPLC. Two brief reports on the use of HPLC for the estimation of mimosine and DHP in *L. leucocephala*, ruminant urine, and chick excreta have appeared recently[35,36]. One of these methods involved the use of phosphoric acid as the ion-pairing agent[35], while the other utilised the ion-pairing effects of sodium octyl sulphate[36].

We report here the detailed methodology of RP-IP-HPLC, using the octyl sulphate anion as the pairing ion, for the estimation of mimosine and DHP simultaneously in *Leucaena* leaf meal (LLM), *Leucaena* seeds, chick excreta and serum.

EXPERIMENTAL

Sample preparation

LLM, Leucaena seeds and excreta. Finely ground samples of LLM, *Leucaena* seeds and freeze-dried excreta (or *ca.* 20 g of a homogenous mixture of fresh excreta) were prepared as described previously[28]. An aliquot of the resultant extract was forced through a Sep-Pak C_{18} cartridge (Waters Assoc., Stockport, Great Britain) to remove or reduce contaminants. Washing the Sep-Pak cartridges with 0.1 *M* HCl (2 × 5 cm^3), methanol (2 × 5 cm^3), distilled water (2 × 5 cm^3) followed by 2–3 cm^3 of extract, prior to collection of the eluate from the Sep-Pak produced a cleaner solution for chromatography. This procedure also allowed re-use of the cartridges.

Serum. Whole chick blood was allowed to stand overnight at 4°C and was then centrifuged (2500*g*) for 8 min. The resultant serum was decanted off and stored at −20°C until required for analysis. Protein precipitation in the serum was accomplished using two precipitants: sulphosalicylic acid (SSA; 8 g in 100 cm^3 of distilled water) and phosphotungstic acid (PTA; $H_3PO_4 \cdot 12WO_3 \cdot xH_2O$; 6 g in 100 cm^3 of distilled water). (Both precipitants were obtained from BDH, Poole, Great Britain). Precipitation of protein was also attempted using both saturated $(NH_4)_2SO_4$ and ethanol, but these proved to be unsatisfactory because addition of SSA to the supernatant precipitated further amounts of protein. Precipitation of serum proteins using SSA and PTA was achieved by adding 0.25 cm^3 of either of the precipitants to 1 cm^3 of serum. The resultant mixture was then centrifuged (3000 g) for 5 min, the super-

natant was decanted off and subsequently forced through a C_{18} Sep-Pak cartridge, pretreated as described above, prior to chromatographic analysis. The Sep-Pak cartridges were then discarded.

Preparation of standards

Stock standard solutions of mimosine (0.25 m*M*) and DHP (0.5 m*M*) were prepared by dissolving the dried, crystalline materials in 0.1 *M* HCl. More dilute standards were prepared by dilution of appropriate volumes of the stock standards with 0.1 *M* HCl. Standard solutions with concentrations in the expected working ranges of 6.25 μM to 0.25 m*M* with respect to mimosine and 12.5 μM to 0.25 m*M* with respect to DHP were prepared. Mimosine (Sigma, Poole, Great Britain) was dried to constant weight in an oven prior to dissolution. DHP was prepared by a modification of the method of Hegarty *et al.*[16]. Mimosine (2 g) was refluxed in 0.1 *M* HCl (200 cm^3) for 24 h, and the resultant DHP separated from other impurities as described by Hegarty *et al.* DHP, however, could not be eluted from the ion-exchange resin using 0.1 *M* HCl (2.5 dm^3). Elution of DHP was accomplished by washing the resin with 1 *M* HCl (700 cm^3). Other preparative details are as described by Hegarty *et al.*[16], although vacuum sublimation was not performed. Repeated recrystallisation of DHP from ethanol followed by washing with diethyl ether yielded buff-coloured crystals (m.p. 240–243 dec; lit. 242–244 dec.[16,37]). Infrared spectroscopy produced a spectrum which was consistent with that expected for DHP, while chromatographic analysis did not reveal any impurities in the prepared DHP. The UV spectrum showed a λ_{max} of 269 nm and an extinction coefficient, in 0.1 *M* HCl, of 516 $m^2\ mol^{-1}$ at 269 nm.

Recoveries

Recovery of mimosine from LLM, estimated using IEC, has been previously reported[28]. Recovery of mimosine, added to excreta prior to extraction, also estimated using IEC, has been found to be 102.7% (± 3.0) (previously unreported results). A comparison of results obtained using IEC with those obtained using HPLC was regarded as yielding sufficient information on recovery of mimosine from excreta, LLM and *Leucaena* seeds. Recovery of DHP was measured by determining the DHP content of LLM and then adding crystalline DHP to the LLM at two levels. Recovery of DHP from excreta was ascertained by adding crystalline DHP to a freeze-dried DHP-free excreta sample. Extraction and subsequent preparation of the sample was as described in the Sample Preparation section of this report. An aliquot of a standard DHP solution was also added to the prepared LLM extract to measure chromatographic recovery, and give an indication of interference caused by any other components in the extract.

Recovery of both mimosine and DHP from chick serum was determined by the addition of 1 cm^3 of a standard solution (0.125 m*M* mimosine and 0.25 m*M* DHP) to 1 cm^3 of serum. Precipitation of protein was accomplished by the addition of 0.5 cm^3 of either PTA or SSA. Further treatment was as described earlier in this report.

Chromatography

An Altex liquid chromatography system (Scotlab Instrument Sales, Lanark, Great Britain) consisting of an Altex Model 110A pump, a Rheodyne 7120 injection valve with 20-μl loop, and an Altex-Hitachi Model 100-10 variable wavelength detec-

tor was used. An Altex column (25 × 0.46 cm I.D.) packed with LiChrosorb RP-18 ($d_p \approx 10$ μm) was used for all chromatographic separations reported here. The column was packed in this laboratory using a Magnus P6050 column packer (Magnus Scientific, Cheshire, Great Britain). Column efficiency, determined using naphthalene eluted isocratically with aqueous methanol (70 cm^3 CH_3OH made to 100 cm^3 with distilled water) at a flow-rate of 1 cm^3 min^{-1}, was 4321 theoretical plates, (*i.e.* $N = 4321$). The buffer used for chromatography was prepared by mixing 200 cm^3 of 0.01 *M* sodium octyl sulphate in 2% v/v HPLC grade methanol (crystalline sodium octyl sulphate was obtained from Kodak, Liverpool, Great Britain and CH_3OH was obtained from Rathburn Chemicals, Peeblesshire, Great Britain) with 240 cm^3 of HPLC grade methanol. Analytical grade sodium nitrate (5.1 g; BDH) was added and the mixture made up to 2 dm^3 with "in glass" double distilled water. The buffer was filtered through a Whatman GF/F glass fibre filter under reduced pressure, and the pH adjusted to 2.25 with HNO_3 (≈ 7.9 *M*). It was then degassed ultrasonically prior to use.

The volume of extracts and standards loaded was restricted to 20 μl.

The methodology used for ion-exchange chromatographic analyses of samples has been previously reported[28].

RESULTS AND DISCUSSION

The excellent linear response of the system to both mimosine and DHP within their respective, expected working concentrations is shown in Fig. 1. Standards with concentrations ranging from 6.25 μ*M* to 0.25 m*M* for mimosine, and from 12.5 μ*M* to 0.25 m*M* for DHP were analysed in triplicate, and peak area was plotted against concentration. Correlation coefficients and standard errors for mimosine and DHP standards, measured at their λ_{max} wavelengths of 278 and 269 nm, respectively, and at different sensitivities, indicate an excellent linear response and precision of analysis for loaded amounts of mimosine from 0.125 nmol to 5 nmol. Loaded amounts from 0.25 nmol to 5 nmol of DHP produced similarly good results.

Typical chromatograms of a standard mixture of mimosine and DHP, those of a 6 *M* HCl extract of LLM and two deproteinised serum samples are shown in Fig. 2. Near-baseline resolution of mimosine and DHP was obtained. The mean resolution (R_S) obtained from nine samples was 1.62 (± 0.106). The relative standard deviation (R.S.D.; 6.54%) is fairly large presumably because the nine samples consisted of serum, LLM, excreta and standards analysed on different days. The resolution was calculated as follows: $R_S = 2\,(t_{R\mathrm{DHP}} - t_{R\ \mathrm{mimosine}})/(W_{\mathrm{DHP}} + W_{\mathrm{mimosine}})$ where $t_{R\ \mathrm{DHP}}$ and $t_{R\ \mathrm{mimosine}}$ are retention times for DHP and mimosine, respectively, and W_{DHP} and W_{mimosine} are base widths of the DHP and mimosine peaks, respectively. The phase capacity ratios (k') of mimosine and DHP were 3.63 (± 0.33) and 5.56 (± 0.493), respectively. The k' values were calculated from the same nine chromatograms used to calculate the resolution.

A small shoulder appeared at the base of the mimosine peak in some excreta extracts, but had little or no effect on the estimation of recovery of mimosine or DHP (Tables I and II). Mimosine or DHP was not detected in the serum of chicks fed a diet containing LLM. An interesting feature of the chromatograms of serum (Figs. 2c and 2d), however, was the concentration of compounds which eluted prior to

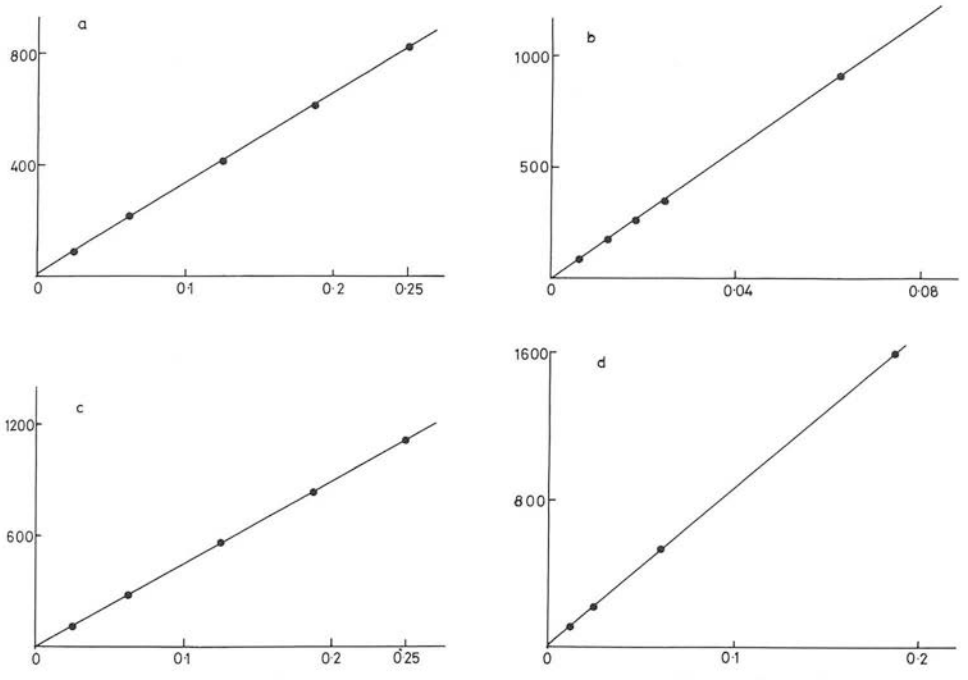

Fig. 1. HPLC response curves for mimosine (a, b) and 3-hydroxy-4(1*H*)-pyridone (c, d) at 278 and 269 nm, respectively. Concentration (m*M*) plotted on abscissa, peak area (mm^2) on ordinate. (a) 0.05 a.u.f.s.: $y = 3271.0x + 6.374$; standard error (S.E.) = 10.3; correlation coeff. (R) = 0.999. (b) 0.01 a.u.f.s.; $y = 14{,}738x - 15.208$; S.E. = 11.2; $R = 0.999$. (c) 0.02 a.u.f.s.: $y = 4486.6x - 0.77$; S.E. = 19.3; $R = 0.999$. (d) 0.01 a.u.f.s.: $y = 8513.5x + 3.57$; S.E. = 10.0; $R = 1.000$.

mimosine. Serum from chicks fed LLM had a higher concentration of these components than had serum from chicks fed LLM-free diets. The identities and significance of these compounds are not known but further study is warranted.

Mimosine values obtained in samples of LLM, chick excreta and *Leucaena* seeds, using IEC, agree well with those obtained using HPLC (correlation coefficient = 1.000) although slightly lower values were obtained for the two excreta samples when determined using HPLC. The percentage R.S.D. values for the seven samples vary somewhat, becoming fairly large when different extracts of the sample were analysed. The R.S.D. values for replicate analyses of the same extract, however, are fairly low even when mimosine was estimated at two different wavelengths. All R.S.D. values are within the limits expected for this type of analysis[38]. The precision, and good agreement with IEC values, obtained for the mimosine content of the LLM sample, analysed at 278 and 269 nm, indicate that no interfering compounds eluted simultaneously with mimosine.

The recovery of DHP (Table II), when added in crystalline form to LLM and excreta, and in solution to extracts of LLM, averaged 98.9%. The excellent recovery of DHP when added to extracts of LLM indicates that no interference from other compounds in the extract had occurred. Losses during clean-up of the sample were also negligible. Recovery of DHP, added in the crystalline form to LLM prior to extraction (Table II), is slightly low being least for the lowest level of addition. The

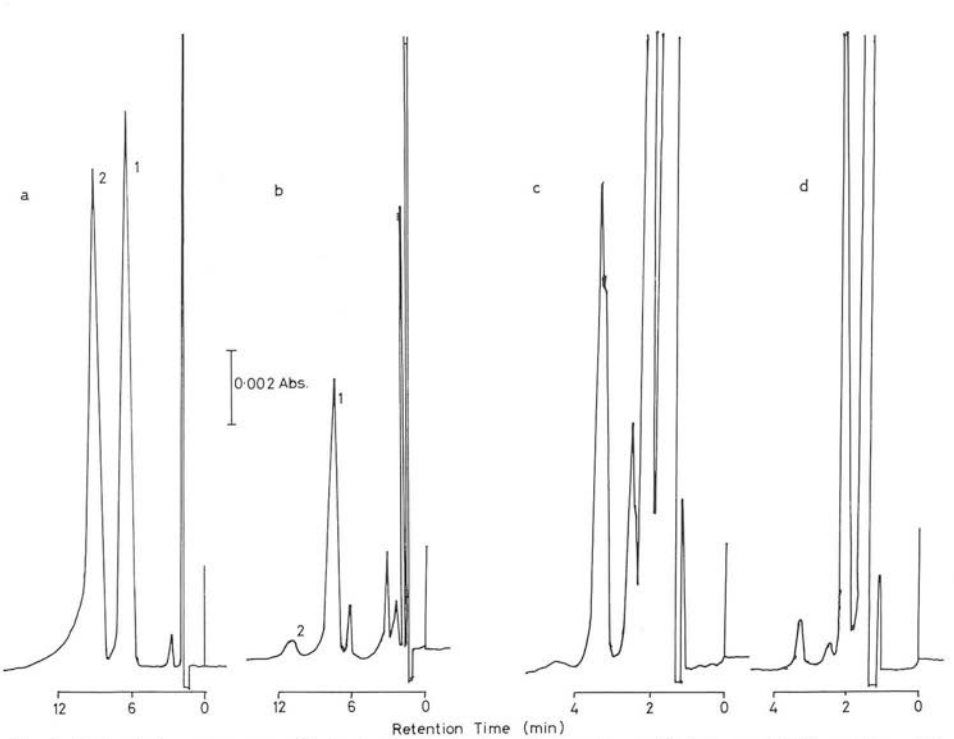

Fig. 2. Typical chromatograms of (a) a standard solution of mimosine and 3-hydroxy-4(1*H*)-pyridone; (b) a *Leucaena* leaf meal (LLM) extract; and serum samples from chicks fed (c) an LLM diet and (d) a diet without LLM. Peaks: 1 = mimosine; 2 = DHP. Flow-rate of pH 2.25 buffer, 1.8 $cm^3\ min^{-1}$; detector 269 nm and 0.02 a.u.f.s.

TABLE I

MIMOSINE CONTENT OF *LEUCAENA LEUCOCEPHALA*, *LEUCAENA* SEEDS AND EXCRETA DETERMINED USING IEC AND HPLC

Sample	*Mimosine concentration (g kg^{-1} dry matter)*		*Percentage R.S.D.*	*HPLC / IEC*
	IEC	*HPLC (± S.D.)*		
Leucaena leaf meal (sun-dried, pelleted) "Peru" cultivar *ex* Malawi 1979	24.27	24.73 (±0.692)*	2.80	1.019
Leucaena leaf meal (sun-dried, unpelleted) "Peru" cultivar *ex* Malawi 1977	10.26	10.42 (±0.260)**	2.50	1.016
Leucaena leaf meal (sun-dried, unpelleted) "Peru" cultivar *ex* Malawi 1979	23.28	23.76 (±0.254)***	1.07	1.021
Leucaena seeds *ex* Mexico 1981	67.54	67.95 (±0.350)§	0.52	1.006
Leucaena seeds *ex* Mexico 1981	73.19	73.93 (±0.052)§	0.07	1.010
Chick excreta (from chicks fed *Leucaena* diets)	3.63	3.43 (±0.027)§	0.79	0.945
Chick excreta (from chicks fed *Leucaena* diets)	1.11	1.01 (±0.007)§	0.69	0.910

* Mean of duplicate analyses of three samples.
** Mean of duplicate analyses of six samples.
*** Mean of four analyses of two samples (two at 278 nm and two at 269 nm).
§ Mean of triplicate analyses of one sample.

TABLE II

RECOVERY OF 3-HYDROXY-4(1*H*)-PYRIDONE (DHP) FROM *LEUCAENA* LEAF MEAL (LLM) AND CHICK EXCRETA (MEASURED AT 269 nm) USING HPLC

Sample	*DHP content by analysis (g kg^{-1} dry matter)*	*Expected DHP content (g kg^{-1} dry matter)*	*Percentage recovery (± S.D.)*	*Percentage R.S.D.*
LLM (sun-dried, unpelleted)* "Peru" cultivar *ex* Malawi 1977 + DHP	3.084	3.069	100.5 (± 2.6)	2.59
LLM (sun-dried, unpelleted)** "Peru" cultivar *ex* Malawi 1977 + DHP	8.484	9.024	98.1 (± 5.4)	5.49
LLM (sun-dried, unpelleted)** "Peru" cultivar *ex* Malawi 1977 + DHP	5.475	5.912	92.6 (± 5.2)	5.64
Excreta (obtained from chicks fed on LLM-free diet)** + DHP	5.197	4.974	104.5 (± 1.3)	1.28

* DHP solution was added to three LLM extracts prior to analysis.

** Crystalline DHP was added to powdered sample prior to extraction. Three samples were taken for extraction of DHP.

R.S.D. values for recovery of DHP from LLM are fairly high, averaging 5.57%. Recovery of DHP from excreta, however, is slightly higher than expected although precision of analysis is good.

Recovery of mimosine and DHP from chick serum (Table III) shows that substantial losses of both mimosine and DHP occur during sample preparation. This is a problem which has been reported[39,41] for analysis of compounds in blood and was not unexpected. Recovery of mimosine was highest when SSA was used as the precipitant with serum levels of mimosine and DHP of 62.5 and 125 nmol cm^{-3}, respectively. Precision of analysis was fairly low however. The use of SSA as the precipitant yielded only *ca.* 58% recovery of DHP at a concentration of 125 nmol cm^{-3}, although precision was fairly good. It was observed that when SSA was used as the precipitant, peak broadening and eventually splitting occurred after the analysis of twelve samples. This condition remained even when standard solutions were subsequently loaded. Removal and replacement of the top 1–2 mm of column packing resolved the problem, indicating that perhaps some proteinaceous material had been adsorbed on to the top of the column. Another possible cause may have been due to adsorption of some SSA on to the column. This possibility, although remote because of the hydrophilic groups on the benzene ring of SSA causing lack of retention, would appear to be confirmed by the fact that peak splitting slowly decreased as the number of injections of a standard solution of mimosine and DHP increased. PTA was used as the preferred precipitant since peak broadening or splitting did not accompany its use. As can be seen (Table III) a reversal in recovery is produced when PTA was used to precipitate the protein from serum containing the same concentrations of mimosine and DHP as those used for SSA precipitation. Precipitation of protein from serum samples containing half the concentration of mimosine and DHP produced an increase in recovery of mimosine but a slight reduction in recovery of DHP. R.S.D. values for the recovery of DHP are fairly consistent but are quite high and variable for mimosine. The combined molar recoveries of both mimosine and DHP are almost identical for both SSA- and PTA-treated serum, although the values obtained for the

TABLE III

RECOVERY OF MIMOSINE AND 3-HYDROXY-4(1*H*)-PYRIDONE (DHP) FROM CHICK SERUM USING HPLC

Serum sample	*Mimosine concentration (nmol cm^{-3})*	*Mimosine recovery (%) (±S.D.)*	*Percentage R.S.D.*	*DHP concentration (nmol cm^{-3})*	*DHP recovery (%) (±S.D.)*	*Percentage R.S.D.*	*Combined recovery (%) of DHP and mimosine*
A*	62.5	81.3 (± 5.5)	6.77	125.0	57.7 (± 1.6)	2.77	69.5
B**	62.5	54.9 (± 1.7)	3.10	125.0	81.8 (± 2.1)	2.57	68.4
C**	31.25	71.1 (± 4.5)	6.33	62.5	75.5 (± 1.2)	1.59	73.3

* Values are the means of triplicate analyses of three samples; sulphosalicylic acid used as protein precipitant.
** Values are the means of duplicate analyses of three samples; phosphotungstic acid used as protein precipitant.

lower concentrations of mimosine and DHP is slightly higher than those for the higher concentrations.

Ion-pairing of mimosine and DHP with the precipitants could have been partially responsible for the low recoveries. If ion-pairing had occurred then it seemed likely that some mimosine and DHP would be eluted simultaneously with either SSA or PTA, both of which were retained only slightly. This premise was tested by adding SSA or PTA to a standard solution of mimosine and DHP (0.125 m*M* and 0.25 m*M*, respectively, in 0.1 *M* HCl) in the same ratio as for precipitation of the protein from serum (*i.e.* 2 cm^3 of standard plus 0.5 cm^3 of precipitant). Analysis of these sep-pak pretreated standards confirmed that losses occurred, possibly by ion-pairing. Recoveries of 85.7% ($\pm$ 0.7) and 87.9% ($\pm$ 1.9) for mimosine and DHP, respectively, were obtained from triplicate analysis of an SSA treated standard. Analysis of a PTA-treated standard showed that recoveries of 78.1% ($\pm$ 0.4) and 85.6% ($\pm$ 0.6) were obtained for mimosine and DHP, respectively. It would appear, therefore, that precipitation of protein from serum using either SSA or PTA causes loss of mimosine by at least two mechanisms: adsorption of mimosine and DHP to the protein, and non-retention of both during chromatography due to ion-pairing with the precipitant. No loss of mimosine or DHP was detected when standard solutions (without added precipitant) were treated with Sep-Pak cartridges prior to analysis. The re-use of Sep-Pak cartridges for LLM and excreta extracts produced cleaner samples than those which had been passed through unused cartridges, without loss of mimosine or DHP.

Recovery of 100.3% ($\pm$ 1.6) has been reported for mimosine added to ovine plasma (in the range 0.5–1.25 μmol cm^{-3}) when analysed using IEC[30]. It appears, however, that this recovery was obtained from samples with mimosine added after protein precipitation with SSA. Losses during protein precipitation would therefore not have been accounted for. We have not yet been able to detect mimosine or DHP in the serum of chicks fed LLM, although levels of up to 0.4 μmol of mimosine cm^{-3} of ovine plasma have been reported for sheep fed diets containing mimosine[9]. Our inability to detect mimosine or DHP in chick serum may be due to a variety of factors, not least of which may be that mimosine or DHP do not enter the blood system. This possibility is likely since it has been reported that a high proportion of mimosine ingested by the chick is excreted[22]. It is also possible that mimosine and DHP, if present in the blood, is bound/adsorbed to protein and thus lost during serum preparation. Different sample preparation techniques, such as hydrolysis of whole blood or ultra-filtration, may yield information on mimosine and DHP in chick blood. Another possibility is that the "known addition" technique could be used to estimate mimosine and DHP in blood and, perhaps, urine. Further work is continuing on this aspect of sample preparation.

A recent report has shown that mimosine and DHP have been separated in 0.1 *M* HCl extracts of *leucaena* and in ruminant urine using RP-IP-HPLC[35]. The authors reported that, when using orthophosphoric acid as the ion-pairing agent, resolution of DHP and DHP-glycoside was not possible. They subsequently resorted to hydrolysis of the urine to convert the DHP-glycoside to DHP. Retention times for mimosine and DHP were of a similar order to those obtained by us although we used a higher effluent flow-rate. No recoveries for DHP or mimosine were given although negligible levels of mimosine were detected in urine. The very low mimosine levels, compared to DHP, appear to be in direct contrast to work with chicks[22,40] (unpub-

lished data) and sheep[9] which has shown that substantial amounts of mimosine and DHP were excreted.

A total of *ca.* 900 samples consisting of LLM, excreta and serum have been analysed in this laboratory, using the same column, since the work presented here was carried out. During these analyses the top 2–3 mm of column packing has been changed five times and, on two occasions, the column was washed with aqueous methanol (70% v/v). The column efficiencies before (901 $\pm$ 25; $n = 4$) and after (996 $\pm$ 34; $n = 4$) the analyses of the 900 samples were almost identical. On both occasions efficiency was determined on two separate days, using the mimosine peak. The long column life indicates that sample preparation was good.

The current study shows that the use of RP-IP-HPLC, using sodium octyl sulphate as the ion-pairing agent, provides a rapid, accurate and precise technique for the estimation of mimosine and DHP in LLM and excreta with minimal sample preparation, using a column of medium efficiency ($N \approx 4000$ measured using naphthalene). Although analysis of both mimosine and DHP in serum by this method gives low recoveries and variable precision, it seems feasible that the technique can be developed with further study and used for the estimation of mimosine, DHP, other metabolites and related analogues in ruminant and non-ruminant blood.

ACKNOWLEDGEMENTS

The authors thank The Tropical Products Institute, London, for their financial support and Dr. R. A. Wall of the Department of Pharmacology, University of British Columbia, Vancouver, Canada for his advice on HPLC.

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SUMMARY OF DIETS FOR EXPERIMENTS 1 TO 8

Leucaena leaf and seed and sorghum

Diet

1.1 LLM 150
1.2 LLM 150 + PEG4000 (20g kg^{-1}).
1.3 LLM 150 + PEG4000 (40g kg^{-1}).
1.4 $Fe_2(SO_4)_3$ (24g kg^{-1}).
1.6 150 + PEG4000 (20g kg^{-1}) + $Fe_2(SO_4)_3$ (12g kg^{-1}).
1.7 LLM 150 + PEG4000 (40g kg^{-1}) + $Fe_2(SO_4)_3$ (24g kg^{-1}).
2.1 soya/maize control
2.2 soya/maize control + $Fe_2(SO_4)_3$ (4.8g kg^{-1}).
2.3 soya/maize control + $Fe_2(SO_4)_3$ (12.0g kg^{-1}).
2.4 LLM 150
2.5 LLM 150 + $Fe_2(SO_4)_3$ (1.6g kg^{-1}).
2.6 LLM 150 + PEG4000 (20g kg^{-1}) + $Fe_2(SO_4)_3$ (1.6g kg^{-1}).
2.7 LLM 150 + $Fe_2(SO_4)_3$ (4.8g kg^{-1}).
2.8 LLM 150 + PEG4000 (20g kg^{-1}) + $Fe_2(SO_4)_3$ (4.8g kg^{-1}).
3.1 soya/maize control
3.2 LLM 150
3.3 LS 65
3.4 LS 65 + $Fe_2(SO_4)_3$ (1.6g kg^{-1}).
3.5 LS 95
4.1 soya/maize control
4.2 LLM 150
4.3 LLM 150 + $FeSO_4$ (1.55g kg^{-1}).
4.4 LLM 150 + $FeSO_4$ (11.9g kg^{-1}).
5.1 soya/maize control
5.2 soya/maize control + PVP40 (10g kg^{-1}).
5.3 LLM 150
5.4 LLM 150 + PEG4000 (20g kg^{-1}) + $Fe_2(SO_4)_3$ (8.5g kg^{-1}) + cholest (5g kg^{-1}).
5.5 LLM 150 + PVP40 (10g kg^{-1}) + $Fe_2(SO_4)_3$ (8.5g kg^{-1}).
5.6 LLM 150 autoclaved
5.7 LLM 150 autoclaved + PEG4000 (20g kg^{-1}) + $Fe_2(SO_4)_3$ (8.5g kg^{-1}) + cholest (5g kg^{-1}).
5.8 soya/maize control + mimosine (3.3g kg^{-1}).
6.1 soya/maize/glucose control + $Fe_2(SO_4)_3$ (8g kg^{-1}).
6.2 LLM 400 + hemicellulase (3.3g kg^{-1}) + $Fe_2(SO_4)_3$ (8g kg^{-1}).
6.3 LLM 400 + hemicellulase (11.9g kg^{-1}) + $Fe_2(SO_4)_3$ (8g kg^{-1}).

7.1 soya/maize control
7.2 sorghum 300
7.3 sorghum 300 + $Fe_2(SO_4)_3$ (8g kg^{-1}).
7.4 sorghum 300 + $Fe_2(SO_4)_3$ (16g kg^{-1}).
8.1 soya/maize control
8.2 soya/maize control + mimosine (3.4g kg^{-1}).

SUMMARY OF DIETS FOR EXPERIMENTS 9 TO 12

jack bean

Diet

9.1 soya/maize control.
9.2 JB 140.
9.3 JB 140 + lysine (6.3g kg^{-1}).
9.4 JB 140 + arginine (6.0g kg^{-1}).
9.5 JB 140 + lysine (6.3g kg^{-1}) + arginine (6.0g kg^{-1}).
10.1 soya/maize control.
10.2 JB 140.
10.3 JB 140 + ornithine (1.5g kg^{-1}).
10.4 JB 140 + ornithine (1.5g kg^{-1}) + arginine (6.0g kg^{-1}).
10.5 JB 140 + ornithine (1.5g kg^{-1}) + arginine (6.0g kg^{-1}) + lysine (1.3g kg^{-1}).
11.1 soya/maize control.
11.2 JB 140, ground prior to autoclaving, dried at 60 C.
11.3 JB 280, germinated 3d.
11.4 JB 280, germinated 5d.
11.5 JB 140, extracted residue.
11.6 soya/maize control + canavanine (3.9g kg^{-1}).
11.7 JB 140, ground prior to autoclaving, dried at 100 C.
11.8 JB 280, ground prior to autoclaving, dried at 100 C.
11.9 JB 280, soaked, minced and aotoclaved, dried at 60 C.
11.10 JB 280, soaked, minced and autoclaved, dried at 100 C.
12.1 soya/maize control.
12.2 JB 140.
12.3 JB 140 + lysine (3.8g kg^{-1}) + arginine (3.6g kg^{-1}).
12.4 JB 140 + lysine (7.5g kg^{-1}) + arginine (7.3g kg^{-1}).
12.5 JB 140 + lysine (11.3g kg^{-1}) + arginine (10.9g kg^{-1}).

lupins

Diet

13.1 soya/maize control.
13.2 lupin 200.
13.3 lupin 400.
13.4 lupin 200 autoclaved.
13.5 lupin 400 autoclaved.
14.1 soya/maize control.
14.2 lupin 400.
14.3 lupin 400 + dry enzymes (1.5g kg^{-1}).
14.4 lupin 400 pretreated with enzymes (1.5g kg^{-1}).
14.5 lupin 400 pretreated with enzymes (3.0g kg^{-1}).